2006

The epidemiology of West Nile virus in Louisiana

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THE EPIDEMIOLOGY OF WEST NILE VIRUS IN LOUISIANA

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy,

in The School of Veterinary Medicine through
The Department of Pathobiological Sciences

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December 2006

ACKNOWLEDGMENTS

There are many people I wish to thank for their help and support in making this project a success. First of all, I would like to thank my committee for guiding and supporting me through this endeavor. I would also like to thank Dr. Robert Truax and Susan Pourciau from the Cell Culture and Media lab for supplying all my Vero cells and cell culture media for the plaque reduction neutralization test. I would also like to thank the entire Louisiana Veterinary Medical Diagnostic Lab, especially Rob Poston for his virology expertise as well as letting me use his lab and to Heather Lampinen for running some of my PCR samples. I am also deeply indebted to Mike Kearney for helping with the statistical analysis. The last group I would like to thank is East Baton Rouge Mosquito Abatement and Rodent Control. They did a wonderful job of collecting samples as well as providing help whenever needed and sharing their arbovirus experiences.

My mom, dad, and sister have always been there for me since the beginning. There are no words to describe how grateful I am to them for everything. I would like to dedicate this dissertation to them and hope they realize my dream could not have been accomplished without their love and support.

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ABSTRACT

West Nile virus (WNV), a member of the genus Flavivirus transmitted by mosquitoes, first appeared in the New York in 1999. Within five years WNV was detected throughout the contiguous 48 states causing disease in reservoirs and accidental hosts alike. In Louisiana, WNV was first detected in 2001 with one human case, ten equine cases, and six dead birds reported. The introduction of WNV into Louisiana presented an unique opportunity to observe an emerging disease unfold, so a study was launched to gain insight into the epidemiology of WNV in Louisiana.

The first component, an environmental predictive model for West Nile virus, was developed using geographic information systems and remote sensing in relationship to the prevalence of human cases and the percent of WNV positive dead birds by parish for 2002 and 2003. Linear regression analysis showed a 13 variable model with environmental and human factors for the 2003 human dataset to be the best model. This model was able to explain 74% of the variation in human WNV prevalence by parish. The results of the model along with one-way chi-square analysis of categorical variables indicated largely urban cycle when the mosquito-bird transmission cycle reaches high levels as the main mode of WNV transmission with spillover to humans, and other accidental hosts.

A serosurvey of wild birds in East Baton Rouge Parish was conducted from November 2002 to October 2004. A total of 1287 samples were tested by the plaque reduction neutralization test. Overall, 222/1287 (17.25%; CI: 15.19-19.31) tested positive. Species, location, sex, age, and monthly differences were detected. The study

identified Northern cardinals (*Cardinalis cardninalis*) as a statistically significant host for WNV in Louisiana.

Mediterranean house geckos (*Hemidactylus turcicus*) were assessed as a potential reservoir for West Nile virus. Geckos were inoculated orally with West Nile virus and a field study was conducted to determine the prevalence of WNV in naturally infected geckos. Results obtained through virus culture and RT-PCR indicated that geckos could become infected with an oral inoculation of WNV, but that naturally infected geckos do not produce high enough viremias to act as a reservoir.

INTRODUCTION

The world has become a smaller place. Advances in transportation have made it possible for a person to circumvent the earth in less than 48 hours. These advances have also created new opportunities for pathogens to infect naïve populations. This was the case with West Nile virus (WNV). Prior to 1999, WNV was an endemic disease in the Middle East and Africa with sporadic outbreaks reported in Asia and Europe.

In 1999, West Nile virus found its way to North America through a still unknown mechanism. Within several years, WNV became firmly established in North America and gradually made its way to Latin America and the Caribbean. It is feared that WNV will eventually make its to South America.

While West Nile virus has caused numerous human, horse, and bird deaths, it has also created an unique opportunity to study the progression of a pathogen in a new niche and has fostered new relationships in human and veterinary medicine. Many studies have been conducted to study the ecology and epidemiology of WNV in the United States as well as other countries in the Western Hemisphere. As a result of these studies, new techniques have been developed to detect arboviruses, animals have been incorporated as sentinels for human disease, and vector surveillance has increased.

The body of work presented here is an attempt to understand the epidemiology of West Nile virus in Louisiana with special emphasis on East Baton Rouge Parish. This was accomplished by building an environmental predictive model for WNV using GIS, examining the seroprevalence of WNV in birds in East Baton Rouge Parish, and exploring the role that Mediterranean house geckos (*Hemidactylus turcicus*) may play in the transmission of WNV.

LITERATURE REVIEW

Arboviruses in Louisiana

Arboviruses or arthropod-borne viruses are maintained in nature in cycles involving hematophagous, or blood-feeding, arthropod vectors and susceptible vertebrate hosts. The virus is transmitted to a vertebrate host during feeding by an infected vector.[1] Accidental hosts such as humans become infected when spillover from the vertebrate host-vector cycle occur. This can occur when there is a rapid, accumulative, and progressive increase in the number of infected and infective vectors and hosts.[2]

In the United States, the first arbovirus isolated was vesicular stomatitis Indiana in 1925.[1] Since then, around 60 other arboviruses have been isolated in the United States. Of these, only members of the families *Togaviridae* (alphaviruses), *Bunyaviridae* (California serogroup viruses), and *Flaviviridae* (flaviviruses) are known to cause significant human encephalitic disease.[1]

Among the alphaviruses, only Eastern equine encephalitis (EEE) and Western equine encephalitis (WEE), cause occasional encephalitis outbreaks in accidental or dead-end hosts such as humans and equines. Birds serve as the primary reservoirs for alphaviruses. In the eastern United States including Louisiana, EEE is the alphavirus responsible for encephalitic outbreaks. EEE has been the cause of epizootic outbreaks in horses since 1831, but wasn't isolated from an equine brain until 1933. The virus wasn't isolated from a human brain in 1938.[1] In addition to horses and humans, EEE has been isolated from birds, small mammals, and reptiles.[1,3] Most mosquito EEE isolates have come from *Culiseta melanura*.[3] EEE is considered the most severe arthropod-borne encephalitis in the United States with mortality rates ranging between 50-70% and those

surviving infection left with mental and physical sequelae. Between 1964 and 2004, the CDC reported 14 human cases of EEE in Louisiana.[4]

The United States is home to several viruses of the California serogroup of bunyaviruses. The most important and widely recognized is LaCrosse (LAC) virus. LAC was isolated in 1964 from a four-year-old girl who died in LaCrosse, Wisconsin.[1] *Aedes triseriatus* is the primary vector associated with LAC. Small mammals are the primary reservoirs for the California-serogroup viruses, which are mainly responsible for encephalitis in children 15 years and younger. LAC infections usually result in recurrent seizures and other sequalae.[5] The CDC has reported 21 cases of confirmed and probable California serogroup (mainly LaCrosse) human encephalitis cases in Louisiana between 1964 and 2004.[4]

St. Louis encephalitis (SLE), a flavivirus, was the third arbovirus shown to be a human pathogen in the United States. It was first recognized as a cause of human encephalitis during an outbreak in Paris, Illinois in 1932, and later isolated from a human sample during an outbreak in St. Louis, Missouri. Historically, SLE has been responsible for the majority of arboviral outbreaks in the United States. SLE has been responsible for more than 1,000 deaths, at least 10,000 severe illnesses, and more than one million mild or subclinical infections.[1] SLE is endemic in several southern states, including Louisiana, and has by far caused the largest number of human encephalitis cases in Louisiana, with 160 cases reported between 1964 and 2004. Seventy-one of these cases were reported during a large outbreak in northern Louisiana in 2001.[4,8] The primary SLE transmission cycle involves *Culex* spp. mosquitoes (*Culex quinquefasciatus* in Louisiana) and birds.[2]

Diagnosing arboviral infections with these viruses can be done using techniques to detect antigen or antibodies. One such technique, direct immunofluorescent antibody (DFA), uses polyclonal or monoclonal antibodies to detect viral antigen and has been used successfully to detect virus in thin sections of patient tissue.[1] Indirect immunofluorescent antibody (IFA), in addition to polyclonal or monoclonal antibodies, requires the use of anti-species antibodies.[1] Antigen capture enzyme-linked immunosorbent assay (ELISA) has been used to directly detect viral antigen in mosquito pools, avian tissues, and human tissues.[4]

Another common technique for virus detection is virus isolation. Virus isolation can be performed in a number of cell lines, including C6/36 (*Aedes albopictus*) or Vero cells, or in suckling mice.[1,4] With virus isolation, isolates must be confirmed by another technique such as ELISA.[4] In the last 10-15 years, newer arboviral tests such as Reverse transcriptase polymerase chain reaction (RT-PCR) and VecTest™ (Medical Analysis Systems, Camarillo, CA) have been developed to detect viral antigen.[9,10] RT-PCR is a sensitive assay that can detect viral RNA in minute quantities while VecTest is rapid immunochromatographic assay used for the qualitative detection of viral antigen.[1,9,10]

Infection with arboviruses can lead to the development of various antibodies, including immunoglobulin M (IgM), IgG (IgY in birds), and possibly IgA. A fourfold or greater increase or decrease in antibody titer between paired acute and convalescent serum samples is required to serologically confirm infection and to rule out infection with other viruses since many of the antibody tests are cross reactive.[1,4] ELISAs depends on the reaction between viral antigens and antibodies to detect IgM or IgG antibodies.[1,4] The ELISA for detecting IgM antibodies, the antibodies which usually indicate recent

infection, is more commonly referred to as IgM antibody capture ELISA (MAC ELISA). The MAC ELISA is relatively easy to perform compared to other antibody tests and can be used to screen a large number of samples.

Another common antibody test for arboviruses is hemagglutination inhibition (HI).[4] HI is used mainly as a screening test since HI antibodies are broadly reactive among viruses of a serogroup. Another method is complement fixation (CF).[4] CF detects antibodies that are complex-specific, short-lived, later to appear, and usually produce a low titer. Birds never produce CF antibodies and some humans produce CF antibodies too late to be of diagnostic value, hence reducing the usefulness of CF for arbovirus testing. Neutralization tests are more sensitive and specific than other antibody tests are considered the "gold standard".[1,4] Neutralization tests are used mainly to the confirm the results of other antibody tests, since they are costly and complex to perform.[4]

Arboviruses and the Environment

Arboviruses are influenced by the environment. In fact, Michael B. Gregg writes, "Environmental determinants such as temperature, rainfall, humidity, and other climatic variables are basic essentials almost always needed in arboviral disease investigations."[11] Environmental variables play a role in arbovirus epidemiology for several reasons. First, the life cycle of these viruses depends on arthropods.[11] A direct influence of precipitation and temperature on vector abundance and survival has been suggested. These two factors affect available water which is a necessary element in the mosquito life cycle. Temperature is also related to the rate of development of mosquito stages, as well as to virus replication and transmission rates.[12] The environment can also cause an effect on vertebrate hosts. For instance, a cool April can delay the nesting behavior of birds,

thereby inducing a synchrony between nonimmune nestling birds and a high abundance of arthropod vectors.[13]

Certain habitats favor arbovirus development and transmission. For instance, the primary habitats for EEE are lowland areas in the eastern half of North America. The microenvironments for these foci are similar and specific. EEE areas are associated with muck-peat soils dominated by hardwoods. Red maple, cedar, and hornbeam are key indicator species for EEE.[3]

Environment also plays an important part in the epidemiology of LaCrosse encephalitis. Many of the mosquito vectors for LAC and the other members of the California serogroup breed in ground pools that may be transient, semipermanent or permanent.[5] Risk factors for LAC encephalitis include residence in or near forested areas, tree holes on premises, and the presence of discarded tires/containers. Hot spots are typically associated with homes constructed on the edges of gullies and ravines, where hardwoods and tree holes are common.[14] For these are the sites favored by *Aedes triseratus*, a major vector for LAC encephalitis.

A comparison between epidemic and nonepidemic SLE years showed deviations from normal monthly temperatures and precipitation.[13] In epidemic years, above-normal temperatures were recorded in January, February, and May through August. Below-normal temperatures were seen in April. January and February were wetter than normal while July had below-normal precipitation. Similar patterns have been seen with Japanese encephalitis.[13]

West Nile Virus Virology

West Nile Virus (WNV) is a member of the family Flaviviridae and the genus Flavivirus. WNV is a member of Japanese Encephalitis virus serocomplex, which also includes Kunjin virus, St. Louis encephalitis, and Murray Valley encephalitis viruses. WNV is a single-stranded, positive-sense RNA virus that is surrounded by a capsid. The capsid is enclosed by a host derived envelope that has been modified by two membrane glycoproteins, E and prM. Glycoprotein E is the most immunologically important structural protein. Glycoprotein E mediates virus-host cell binding, elicits most of the virus neutralizing antibodies, and acts as the virus hemagglutinin. WNV can be divided genetically into two lineages. Lineage I strains have been isolated from Africa, India, Europe, Asia, and North America. These WNV strains have been associated with avian and human morbidity and mortality. Lineage II strains have only been isolated from Africa and have not been associated with human encephalitis cases.[15]

West Nile Virus and Humans

WNV was first isolated from a febrile woman in the West Nile region of Uganda in 1937.[16] In 1942, shortly after WNV was first isolated, it was shown to be closely related to SLE virus. The first reported human epidemic occurred in Israel in 1951, and the first isolation of WNV from *Culex* spp. came in 1952.[16]

WNV infections usually result in asymptomatic or mild disease. It is estimated that less than 1% of the people infected with WNV develop encephalitis or meningitis.[6,15] WNV infection can cause mild symptoms, such as fever, headache, and rash, or more severe clinical signs, including altered mental state, confusion, seizures, photophobia, gastrointestinal complaints, and muscle weakness.[6] Laboratory findings may include

slightly increased sedimentation rate and a mild leukocytosis. Cerebrospinal fluid is usually clear with a moderate pleocytosis and elevated protein.[17,18]

Most of the early research into the epidemiology of WNV was carried out in the upper Nile Delta. Research studies included serological surveys in humans, mammals, and birds; isolation of virus from naturally infected hosts and vectors; experimental infection in humans, equines, birds, and arthropods; and ecological studies. These studies showed that the primary cycle involved birds and mosquitoes, while humans and equids were accidental hosts. Serological testing and virus isolation from humans in the early 1950's showed that WNV was endemic in parts of East Africa in southern Sudan, and down into the Nile Valley in Egypt. Serological testing and virus isolation also demonstrated that WNV was a disease of early childhood. In children, WNV is primarily a febrile illness associated with the summer months, with encephalitis being a rare clinical manifestation of infected patients in endemic areas. In epidemic situations, central nervous system involvement occurred more frequently, especially in elderly patients.[18]

Experimental infection with WNV was performed in patients with incurable cancers in New York City.[19] Patients were injected with high doses of WNV intramuscularly or intravenously. Experimentally infected patients developed mild clinical disease, including fever similar to naturally occurring infections, and had detectable virus in their blood as soon as 24 hours post-inoculation which lasted for 6 days or more.[19]

Outbreaks of WNV in Israel in the early 1950's also provided vital information about the epidemiology of the disease. Like Egypt, children represented most of the

cases that were infected with WNV. The main clinical signs included exanthema, headache, myalgia, anorexia, abdominal pain, and vomiting. Patients with severe neurological signs were not reported until 1957.[19]

The first indication of WNV in Europe came in 1958 when neutralizing antibodies were detected in two Albanian citizens.[18-20] The first WNV cases in Europe occurred in France in the summer of 1962. Several encephalitis cases were reported in Camargue and Languedoc. It wasn't until September 1964 that WNV was isolated from *Culex modestus* and two entomologists in France. During 1963 and 1965, a serosurvey showed the prevalence of WNV to be between 21%-41% in humans sampled.[7] Between 1974 and 1979 another serosurvey conducted in Camargue showed the prevalence to only be 4.9%.[19]

Since the outbreak of WNV in France, there have been several other European outbreaks, including Spain and Russia in the 1960's, Ukraine and Belarus in the 1970's, and Ukraine again in 1985.[18,20] Serosurveys conducted in Spain during the 1970's showed varying degrees of WNV exposure, ranging from 8% in eastern Spain to 30% in one village.

In South Africa, the first evidence of WNV was documented in 1958. Field studies conducted in the late 1960's and early 1970's showed the incidence of WNV in adults in Karoo and the Orange River to be 13% and the incidence in adults in Cape Province to be 24%.[19] Children, on the other hand, only had an incidence of 5%. In 1974, a large epidemic of WNV occurred in Karoo and northern Cape Province after a heavy rainy season and an increased density in the main vector, *Culex univittatus*. The outbreak lasted from January to June, with peak cases occurring between February and

April. Three thousand cases were reported, but estimates through serosurveys and clinical reports suggested that there were as many as 18,000 human infections.[18,21]

WNV has also made its presence known in Asia. In the 1960's, WNV was isolated from patients in Lahore and Rawalpindi, Pakistan.[22] Between 1978 and 1979, a serological survey for WNV antibodies conducted on residents living in Chiniot and Changa Manga National Forest areas of Punjab Province, Pakistan, showed neutralizing antibodies in 32.8% and 38.5% of those tested, respectively.

Beginning in the 1990's WNV outbreaks with recorded neurological manifestations were becoming more common. Between August and September 1994, an epidemic occurred in the Timimoun oasis in central Sahara, Algeria.[18,19] Almost 50 cases presented with high fever and neurological signs. Among these cases were 20 patients with encephalitis, of whom 8 died.

The first WNV outbreak in a predominantly urban setting occurred in Bucharest, Romania between July and October, 1996.[18-20] Eight hundred and thirty-five people were hospitalized with suspected CNS infections. Of these, 17 cases were fatal, and all the patients were over 50 years old. WNV testing demonstrated antibodies in 393/509 (77%) of the patients sampled. Smaller WNV outbreaks continued in Romania through 2000, with several additional fatalities. Prior to the 1996 epidemic, human serosurveys showed the prevalence of WNV antibody to be between 2-18% indicating WNV activity prior to the epidemic.

Russia experienced a WNV outbreak in August and September 1999 in the Volgograd region.[19,20] The total number of suspected WNV cases was estimated to be 480 people, with many of them experiencing encephalitis. Forty deaths were associated

with this outbreak, and the majority of the cases (75%) were over 60 years of age. Previous WNV outbreaks in the late 1960's in Russia saw a high number of seropositive people, but only 10 confirmed cases of WNV encephalitis.

Other countries that have detected WNV antibodies in human sera include Armenia, Borneo, China, Georgia, Iraq, Kenya, Lebanon, Malaysia, the Philippines, Sri Lanka, Sudan, Syria, Thailand, Tunisia, and Turkey.[18-20] The human distribution of WNV alone makes this virus one of the most widely distributed flaviviruses in the world.

West Nile Virus and Birds

Preliminary research indicated that domestic animals did not produce a viremia large enough to infect the number of feeding mosquitoes necessary for widespread infection in endemic WNV regions.[19] This led to the suspicion that wild birds serve as reservoirs for WNV. One of the first pieces of evidence for this came from the isolation of WNV from the brain, spleen, and blood of a sick pigeon in July 1953 from the north central area of the Nile Delta.[16,23,24]

Since the isolation of WNV from that pigeon, many studies and experiments have been performed to identify possible reservoir species as well as to provide insight about the role wild birds play in the epidemiology of WNV. Ecological studies demonstrated that seropositive humans were correlated with seropositive crows.[16] Work et al. conducted one of the earliest experiments in the mid-1950s in Egypt involving birds.[16] Neutralizing antibodies were found in all common avian species tested from an endemic area. Hooded crows had a high incidence of neutralizing antibodies, especially in endemic WNV areas, as well as a 100% mortality rate in experimentally infected crows. These authors speculated that the 100% mortality rate in experimentally infected crows

could imply a high mortality in naturally infected crows, but they also stated that many survived as indicated by the high percentage of antibody positive crows. The authors also found that hooded crows and sparrows had high enough viremias to complete a bird-mosquito-bird transmission cycle. They were also able to infect birds with WNV infected *Culex pipiens* and *Culex univittatus* mosquitoes.[16,23,24]

In a study involving wild South African birds, 13 species from 5 different families infected in the study developed viremias capable of infecting mosquitoes.[21] Virus was detectable for as long as 4 days. Thirty days after infection, WNV antibodies were detected in 92% of the birds by hemagglutination inhibition and in 70% of birds by virus neutralization. Another group of researchers also reported WNV antibodies lasting for 18 months in Laughing doves following virus inoculation.[25]

Additional international studies have shown that birds play an important role in the dissemination and maintenance of WNV in the environment. One study in Slovakia tested 169 birds from 35 species between 1971 and 1973 from the Záhorská lowland, the basin of the Ipel River and East Slovakia.[26] Antibodies against WNV, as well as the virus itself, were found in several species of migratory birds. The authors commented about isolating WNV from migratory birds and the importance of migratory birds in dissemination of WNV throughout Eurasia as well as the geographic range of WNV in temperate, subtropical, and tropical zones.

Between October 1962 and September 1965, 2,022 blood samples were collected from 17 families and 51 species of birds at Olifantsvlei in South Africa.[21] All samples were tested for WNV antibodies. In the study, 252 (12%) of the birds tested positive for WNV antibodies by hemagglutination inhibition. In contrast, a prevalence of 53% in 322

avian samples tested was obtained shortly following one of the largest WNV human outbreaks in 1974 in Karoo, South Africa.[18,21,25]

WNV infected birds have also been found in Asia. In 1978-79, 317 birds representing 32 different species were captured in the Chiniot and Changa Manga National Forest areas in Pakistan.[22] Using plaque reduction neutralization tests, 85 (26.8%) of the birds representing 21 species were antibody positive. One bird that was initially antibody positive was antibody negative when it was recaptured 26 days later. WNV neutralizing antibodies have also been found in wild birds samples in India.[27]

In 1985, an enzootic WNV cycle was detected in Czechland.[18,20] WNV antibodies were identified in 4% of 704 free-living wetland birds. Seventeen hatchling year wetland passerines of 7 species were among those testing positive. Three years later, a serosurvey showed that 29% of 110 sentinel domestic ducks kept on the same pond seroconverted.

In 1996, the first year in a series of WNV outbreaks in Romania, wild bird and domestic fowl serum was submitted for WNV antibody testing via plaque reduction neutralization against the WNV Eg101 strain.[28] Only one wild bird sample, (*Erithacus rubecula*), tested positive, while 30/73 (41%) domestic birds tested positive. The following year saw the dispersion of 160 sentinel chickens in and around the city of Bucharest.[18-20,23,28] Chickens were bled biweekly between June 26 and August 13, and again on October 16. Chickens showed evidence of WNV activity at every sampling period and even demonstrated WNV activity before the first human case was reported. Adult chickens are routinely used as sentinels since they are refractory to WNV infection.

Many of the previously mentioned avian studies have reported limited mortality or morbidity associated with the detection of WNV, and infectivity trials have shown that birds are highly susceptible to WNV infections, but they rarely show clinical signs. In Israel in November 1997, young domestic geese from four flocks presented with acute neurological signs of paresis along with high morbidity and mortality.[23]

In September and October of 1998, WNV was isolated from a flock consisting of primarily fledgling white storks (*Ciconia ciconi*) that arrived in Eliat, Israel, from Europe on August 26, 1998.[23,29] Four WNV isolates were obtained from brain tissue of storks that were dead or close to dying two days after their arrival. Neutralizing antibodies against WNV were also detected in three birds six days after their arrival. These results indicated that the birds had been exposed to WNV in Europe prior to their migration southward. This view was further strengthened by the presence of neutralizing antibodies in storks in Germany.

West Nile Virus in Equine Hosts

Horses seem to be one of the most susceptible accidental hosts for WNV, along with man. Early experimental studies of horses fed on by WNV infected mosquitoes found that the horses developed neutralizing antibodies, but not viremias.[24] One of the first reported WNV infections in a horse occurred in a 12-year-old police horse in Egypt in 1959. The horse died and was necropsied. Necropsy findings included severe congestion of the surface of the cerebrum, congestion of the urinary bladder with petechial hemorrhages on the mucous membranes, moderate congestion of the stomach and small intestine, and foci of congestion in both lungs. WNV was isolated from brain tissue.

Also during this time in Egypt, a serosurvey for WNV in equids was conducted between January and May 1959.[19,24] In this study, 436 samples were collected from horses, donkeys and mules from both Upper and Lower Egypt. The prevalence of WNV neutralizing antibodies was 67% in horses, 47% in donkeys, and 44% in mules. The prevalence in equines aged 6 years or older ranged from 14% in Alexandria to 89% in Qena.

In 1962, WNV was first reported in France in domestic and free-living horses.[18,19,24] WNV was associated with nervous system abnormalities in both of these groups. It is estimated that 20-30% of the 50 reported cases in domestic horses were fatal. Three additional cases were reported in 1965.

In Morocco, an outbreak of WNV happened in 94 horses, donkeys, and mules from August to November 1996.[19,24] Forty-two of these cases were fatal. No breed predilections were detected and all age groups were affected. WNV was isolated in cell culture from this outbreak.

The first recorded outbreak of WNV in horses in Italy occurred in 1998.[18,19,24] Fourteen horses were confirmed as having WNV infection. All the horses had evidence of ataxia and hind limb weakness. Six horses had clinical signs that progressed to paraparesis, tetraplegia, and recumbency within 2-9 days. Two of the horses died while the other four were euthanized. Pathology findings showed a mild to moderate, nonsuppurative polioencephalomyelitis with regular involvement of the ventral horns of the thoracic and lumbar spinal cord. In affected stables, the morbidity rate varied between 0.4% and 20%. It was also reported that 39.6% of 159 local horses examined had WNV antibodies.

West Nile Virus and Other Species

In other domestic animals, experimental infections have shown that domestic animals rarely develop viral titers or clinical signs after being exposed to WNV. In one study, sheep that were fed on by WNV infected mosquitoes failed to become viremic, but one sheep did develop neutralizing (NT) antibodies.[24] Experimental studies with pigs have shown them to be poor hosts for WNV. Experimental infection of calves failed to produce a viremia. Dogs that were inoculated with WNV subcutaneously and intravenously developed HI and NT antibodies. One of the inoculated dogs developed a low titer viremia.[24,30]

Other domestic animals can develop WNV antibodies in natural settings. In eastern Slovakia, WNV antibodies were found in 1.0% of 608 sheep tested.[18,24] Another prevalence study in Romania from the 1970's found that 4.9% of sheep, 4.1% of cattle, and 12% of goats had HI antibodies against WNV.[18,19,24] In Madagascar, 33% of the oxen tested in Mandoto and 2% of the oxen tested in Tsiroanomanddy had WNV antibodies.[24] A survey of dogs in the Orange Free State of central South Africa revealed that 174/377 (46%) of the dogs had HI antibodies against WNV and WNV has been isolated from one of the dogs that was HI negative.[24,30] In Romania, the prevalence of WNV HI antibodies was up to 45% in dogs and 2-16% in cattle, goats, and rabbits prior to the human epidemic in 1996.[20] In Bareilly, Uttar Pradesh, India, HI antibodies were detected in 6.8% of goats, 1.2% of cattle, 27.7% of pigs, 25.9% of horses, 13.8% of water buffalo, and 24% of dogs. Of 389 pigs observed in Kolar district, Kamataka state, India, 10 of them seroconverted to WNV and one produced a WNV viral isolate. In Pakistan, a

serosurvey of 58 Indian cows between 1978 and 1979 revealed 21 cows with antibodies to WNV.[24]

Several studies have conducted to determine if wild mammals play a role in the epidemiology of WNV. Many of these studies have been carried out in primates. In experimental WNV infections in monkeys, the virus persisted for extended periods of time in asymptomatic, pyrexic, or encephalitic monkeys.[24] Intracerebral infection in rhesus macques (*Macaca rhesus*) resulted in 60.7% (34/56) developing encephalitis and seven fatalities.[24] Researchers were also able to infect Bonnet macques (*Macaca radiata*) via intranasal inoculation. Lemurs (*Lemur fulvus*) experimentally infected with WNV produced viremias sufficiently high to infect *Aedes egypti*, but did not develop clinical signs. In Haut Uele in the Democratic Republic of Congo, 37% of 94 chimpanzees had antibodies to WNV. In Madagascar, serological surveys found 1.9% of 377 lemurs and 11.4% of 96 bats (*Pteropus rufus*) had antibodies to WNV.[24]

In other wild mammal studies, a young water buffalo fed on by two WNV infected mosquitoes failed to produce a detectable viremia, but a seroprevalence study found 72% of the water buffalo sampled had WNV neutralizing antibodies.[24] In the same seroprevalence study, 78% of camels sampled also had neutralizing antibodies to WNV. WNV has also been isolated from camels in Kano, Nigeria.[24] WNV was isolated from two rodents (*Arvicanthis nilticus*) in West Africa between 1966 and 1970.[24,31] A study carried out in Israel found the prevalence of WNV HI antibodies in domestic livestock to be 37%, 11% in rodents, and 7% in bats.[24]

In Europe, WNV antibodies have been detected in wild rabbits (*Oryctolagus cuniculus*), brown bears (*Ursus arctos*), wild boars, and hedgehogs.[20] HI antibodies

against WNV were detected in 16.9% of game animals including Roe deer, Red deer, Fallow deer, Mouflon, wild boar, and brown hare in Monrovia, Czechoslovakia. Small mammals at waste disposal sites in Austria were found to have WNV antibodies.[20]

Another study also found WNV antibodies in a reptile, (*Natrix natrix*), from Austria.[20] In contrast, the lake frog (*Rana ridibunda*) in Russia appears to be a competent reservoir for WNV.[18,24] In Hungary, WNV was isolated from two wild rodents. In the Nile Delta, 11.5% of 261 peridomestic rodents tested had WNV antibodies.[24] It is still unclear what role wild mammals play in the epidemiology of WNV, but it is speculated that they are accidental hosts like humans.

West Nile Virus and Vectors

To be incriminated as an arbovirus vector for human disease, an arthropod must feed on humans under natural conditions, show a biological association in time and space with the occurrence of clinical or subclinical human infections, the pathogen of concern must be isolated from the arthropod, and the arthropod must demonstrate under controlled conditions efficient transmission of the pathogen to a suitable host.[12]

Experimental transmission was first documented in *Aedes albopictus* in 1942.[32] By 1950, experimental transmission was also reported in *Culex pipiens* and *Culex tritaeniorhynchus*. Experimental trials in conjunction with field studies from 1952-1954 in the Sindbis sanitary district of Egypt showed conclusively that mosquitoes were the vectors of WNV. Isolates were obtained during this study from *Culex antennatus, Culex univittatus,* and *Culex pipiens.*

In Egypt, Israel, and South Africa, *Culex univittatus* has been implicated as the primary WNV vector based on field isolation rates.[32] In Egypt, nine isolates from

mosquitoes were obtained at the same time as isolations from febrile children.[32] South African isolates have also come from *Culex theileri, Culex pipiens, Culex neavei, Aedes caballus, Aedes circumluteoulus*, and *Coquillettidi* spp. WNV was isolated in Africa from *Culex poicilipes* in Senegal and *Aedes albocephalus* in Madagscar.[18]

In southwestern Asia, the members of the *Culex vishnui* complex including *Culex vishnui, Culex triaeniorhychus*, and *Culex pseudovishnui* are the main vectors for WNV based on studies in Pakistan and India.[32] In Europe, WNV has been isolated from *Culex modestus*. *Culex pipiens* was confirmed as a WNV vector in Europe during the 1996 WNV epidemic in Romania.[28,33]

Ticks have also been implicated as vectors of WNV although their importance is unclear. Soft ticks, *Argas hermanni* and *Ornithodoros capenisis,* from Egypt and Azerbaijan, respectively, have yielded WNV isolates.[18] WNV has also been isolated from hard ticks of the genera *Hyalomma, Rhipicephalus, Amblyomma*, and *Dermacentor* in Astrakhan, Azerbaijan, Turkmenistan, Central African Republic, and Moldavia.[18] Experimental transmission has been reported in *Argas hermann*i, *Ornithodoros capensis,* and *Dermacentor marginatus.*

The History of West Nile Virus in the Western Hemisphere

In 1999, an outbreak of human encephalitis in New York City was initially diagnosed as SLE virus.[34] This initial diagnosis raised a number of issues that could not be explained: 1) SLE has been historically absent from New York City, 2) there were no other outbreaks of SLE at the time, 3) the infected patients experienced profound muscle weakness, 4) labs had a difficult time obtaining a definitive serological diagnosis, and 5) an increase in dead birds was observed, specifically crows and other corvids.[34]

The initial encephalitic patients were diagnosed for SLE using a serological screening test, immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (MAC-ELISA).[34] The MAC-ELISA exhibits cross-reactivity among closely related flaviviruses. Further laboratory testing using the plaque reduction neutralization test were inconsistent with the MAC-ELISA results. Acute and convalescent sera testing with MAC-ELISA and PRNT confirmed the discrepancy.

At the same time as the human encephalitis outbreak, numerous wild birds including American Crows (*Corvus brachrhynchos*) and several other species were found dead across New York City.[24,35-37] Captive birds at the Bronx Zoo, including guanay cormorants (*Phalacrocorax bougainvillei*), bald eagles (*Halieaeetus leucocephalus*), bronze-winged ducks (*Anas specularis*), snowy owls (*Nyctea Scandia*) and several other species died during this outbreak in New York. Necropsy findings from the 27 birds from the zoo revealed gross brain hemorrhage, splenomegaly, meningitis, encephalitis, and myocarditis.[2]

In September 1999, a veterinarian from the Bronx Zoo sent samples to the U.S. Department of Agriculture's National Veterinary Services Laboratories (NVSL) in Ames, Iowa.[36] NVSL identified a flavivirus as the causative agent. NVSL contacted the Centers for Disease Control and Prevention (CDC) for further testing. Samples from three of the encephalitic patients were sent to the University of California at Irvine where a WNV-like virus was identified. On September 24, 1999, the CDC, as well as the U.S. Army Medical Research Institute of Infectious Diseases confirmed WNV in the samples.[17,36] WNV serology testing on banked sera from the Bronx Zoo confirmed that the virus was not in circulation in the zoo prior to the summer of 1999.[37]

WNV was isolated from multiple organs including kidney, heart, intestines, brain, spleen, lungs, ovaries, liver, adrenals, and pancreas from infected birds. Full length genomic nucleotide sequencing of early isolates of WNV from a Chilean flamingo (*Phoenicopterus chilensis*) at the Bronx Zoo and a horse have shown that the strain present in the United States most closely resembles the WNV strain isolated from Israel (1998) with homogeneity of 99.8% over a 1278 nucleotide base region of premembrane (prM) and envelope (E) proteins.[6,7,15,38]

Autopsies were performed during the outbreak on four WNV human patients who suffered clinically from fever and profound muscle weakness.[39] Two of the cases had encephalitis while the other two were diagnosed with meningoencephalitis. The inflammatory response was primarily comprised of mononuclear cells, with formed microglial nodules and perivascular clusters in both white and gray matter. Brain stem involvement was also noted. Two of the brains examined had endoneural mononuclear inflammation of the cranial nerve roots. The only other significant finding was pancreatitis in one of the victims.

Twenty-five horses were reported to be affected by the initial outbreak of WNV in 1999.[6,24] Clinical signs in the horses included ataxia, wide-based stance, hypermetric gait, staggering, stumbling, leaning or circling, head tilt, toe dragging, and behavioral changes. In severe cases, muscle fasciculations and tremors of the face, lip, trunk, or shoulder were reported. The case fatality rate for horses in New York was 25%.

At necropsy, gross lesions were often absent in these horses, but thickened and adherent dura as well submeningeal edema with hemorrhage were reported.[6,24] Histopathology findings included mild to moderate nonsuppurative encephalitis and

vasculitis with monocellular infiltration, perivascular cuffing, and multifocal necrosis. A serosurvey of horses found on the same premises as the clinical cases found 23% of the horses to be seropositive compared to only 8% of horses stabled on the premises that did not have clinical cases.

By the end of 1999, WNV activity was detected in other parts of New York, New Jersey, Connecticut, and Maryland.[6,7] This number quickly grew to eight northeastern states in 2000. In 2000, 21 human cases, 63 equine cases, and six other mammal species were infected with WNV. In addition, the CDC reported that 4304 dead birds and 480 mosquito pools tested positive for WNV. In 2001, there were 66 WNV cases, 64 of them classified as neuroinvasive cases, and 612 mosquito pools were shown to be positive for WNV in a total of 27 states and the District of Columbia. The year 2001 also saw the introduction of WNV on alligator farms and the spread of WNV into Canada.[6,7,17,24,40-42]

In 2002, WNV had spread westward to include 44 states and the District of Columbia.[43] Over 4100 human cases were reported to the CDC. The year 2002 also saw the introduction of WNV to Mexico, Jamaica, Guadeloupe, and the Dominican Republic.[43-49] There were reports of WNV transmission through blood transfusions, organ transplants, intrauterine transmission, and the report of possible transmission through breastfeeding. The high number of WNV human cases in 2002 in the USA surpassed the previous record of 3000 cases set in Karoo, South Africa in 1974.[18,19,21,32]

After 2002, WNV continued its westward expansion. In 2003, the states in the western plains, and the front range of the Rocky Mountains, reported the majority of WNV cases.[42] By the end of 2004, WNV was detected in all of the 48 contiguous states. Between 1999 and 2004, 16,706 WNV cases and 666 deaths were reported to the CDC.

Of these cases, 7,096 were classified as neuroinvasive, 9,268 were classified as West Nile fever, and 342 cases had other or unspecified clinical presentations. By 2005, these totals had increased to 19,525 WNV cases and 771 deaths. The CDC estimates there are approximately 150 people infected and 20 with West Nile fever for every reported WNV neuroinvasive disease case, and thus the number of people asymptomatically infected with WNV represents a much larger number.[4,42]

Since the introduction of WNV into the United States, the virus has become nearly ubiquitous. This can be attributed to the multiple species that can act as reservoirs and vectors. WNV has been found in almost 300 native, exotic, and introduced bird species.[50] Despite the high number of bird species that have tested positive for WNV antibodies or antigen, only a handful have been evaluated as amplifying hosts for the virus. The role of most of these birds in the transmission and dissemination of WNV is still unknown, but evidence suggests that passerines have emerged as the most important amplifiers of the virus.[51]

WNV has been detected in sixty mosquito species of the genera *Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, *Culiseta*, *Deinocerites*, *Mansonia*, *Orthopodomyia*, *Psorophora*, and *Uranotaenia* by viral isolation, RT-PCR, or other antigen detection techniques.[4] Many of the isolates from the initial outbreak in New York were from *Culex pipiens*. Early experiments demonstrated *Culex pipiens* could become infected after ingesting a blood meal with a high viral load, but was considered an insignificant vector due to the low number of field isolates prior to its introduction into the United States.[32] In the U.S., the primary vectors for WNV cycles are *Culex* spp. with great regional variation. In the northeast, *Culex pipiens* is the primary vector while in the southeast,

Culex quinquefasciatus has emerged as the main vector. As WNV continued west in 2003, *Culex tarsalis* was recognized as the most important vector in the Plains and Rocky Mountain regions, and *Culex quinquefasciatus* once again emerged as the most important species in the WNV epidemics of the southwestern United States in 2004.[42]

The United States experience with WNV has shown many other species besides birds, horses, and humans may be affected by WNV. The CDC has reported WNV infections in over 30 mammalian species, including eastern chipmunks (*Tamias striatus*), striped skunks (*Mephitis mephitis*), sheep (*Ovis domesticus*), harbor seals (*Phoca vitulina*), rabbits (*Oryctolagus cuniculus*), white-tailed deer (*Odocoileus virginianus*), black bears (*Ursus americanus*), and big brown bats (*Eptesicus fuscus*).[50,52-55] In 2002, a wolf pup (*Canis lupus*) and a dog from Illinois were diagnosed with WNV encephalitis and myocarditis.[56] Three Eastern fox squirrels (*Sciurus niger*), as well as 13 gray squirrel (*Sciurus carolinensis*) suffered from neurological abnormalities due to WNV.[57,58] Four reindeer at the National Animal Disease Center (NADC), Ames, Iowa were also infected with WNV.[59] Among reptiles, it has been shown that alligators (*Alligator mississippiensis*) could act as amplifiers of WNV.[40,41,60]

The history of WNV in the United States is consistent with the pattern of outbreaks in the Middle East and Europe: 1) an increase in the frequency of outbreaks in humans and horses, 2) high avian death rates accompanying the human outbreaks, and 3) an apparent increase in severe human disease with mortality rates ranging from 5-14% in patients with neurological signs.[15]

Because of the high wild bird mortality rate, dead bird surveillance is being used as an indicator of WNV activity and a determinant of risk for humans in particular areas.

Dead birds are currently used as an early warning system, since WNV positive dead birds often precede human cases. In New York, dead crow density has been shown to be a factor intimately associated with human cases. [61-64]

West Nile Virus and Climate

Climate influences the transmission of WNV because it affects the distribution and abundance of mosquito vectors that transmit the virus and also the extrinsic incubation period, and the development rate of WNV in mosquito hosts.[13] These effects are complex however, for example, a slight increase in rainfall can increase vector density by creating ground pools and other breeding sites for a vector while heavy rain can have a flushing effect that causes a cleansing eggs, larvae, and pupae from a site. Droughts may eliminate standing water, but may cause flowing water to become stagnant. High temperature can increase the abundance of the vector by accelerating egg laying. Previous studies have indicated that hot, dry summers may promote WNV outbreaks in humans.[65]

The extrinsic incubation period, or the time from when a mosquito acquires an arbovirus to the time the vector transmits the virus, is also affected by temperature because mosquitoes are poikilotherms.[66-69] Increasing temperatures shortens the extrinsic incubation time for a virus. In essence, a shorter period of time is required to produce a viral load capable of causing infection. Higher temperatures also shorten the mosquito life span.

A laboratory study with *Culex univittatus* that were infected orally with WNV revealed that it took 58 days for mosquitoes held at 14ºC to reach a mean viral load of 7.0 $\log_{10}$ CPD_{50}/ml of mosquito suspension compared to 15 days for mosquitoes held at

30ºC.[70] The study also showed that took less time to reach 100% transmission rate for mosquitoes kept at 30ºC than at lower temperatures.

The effect of overwintering temperatures on WNV infected *Culex pipiens* has been studied.[71] Mosquitoes were allowed to feed on leghorn chicks that were inoculated with 10^4 PFU/ml. Virus was recovered from most mosquitoes held at 26°C. In addition, mosquitoes transferred to 26°C after being held at lower temperatures showed an increase in infection and dissemination of WNV upon being transferred to 26°C. Studies with other flaviviruses have shown similar results indicating that warmer temperatures are more likely to favor flavivirus replication and transmission.[13] This is why temperature is an important climatologic and remote sensing component for analysis.

West Nile Virus and Geographic Information Systems

Geographic Information Systems (GIS) and remote sensing can be used to examine the relationship between pathogens, their vectors, and environmental components such as habitat and climate. GIS and remote sensing are currently being utilized to study WNV as it continues to sweep across the United States.[72-74]

GIS has been used to develop WNV risk models in New York City where researchers developed the Dynamic Continuous-Area Space-Time (DYCAST) system to identify and monitor high-risk areas for the virus.[75] The DYCAST risk assessment was based on a geographic model that uses a localized Knox test to capture nonrandom space-time interaction of dead birds. The model was built based on 2000 WNV positive dead birds and human cases. Implementation of the model in 2001 identified areas of high WNV risk in five of seven cases, at least 13 days in advance.

In Chicago, another study evaluated the environmental and social determinants of human health risk during the WNV epidemic of 2002.[74] The epidemic in Chicago included over 680 human cases that were clustered in two well-defined areas. The important risk factors included in the model were the presence of vegetation, age, income, race of human population, distance to a WNV positive bird, age of housing, presence or absence of mosquito abatement, and geologic features. These factors explained 53% of the variation in the WNV clusters.

In a study in Indiana, WNV data from serologically confirmed WNV horses were used to develop a temperature-dependent, spatial model of disease dynamics.[76] Cases were examined from August to October 2002 at 108 locations in northern Indiana based on daily maximum temperature from meteorological stations in the area. Results indicated the epidemic was predicted to occur three days earlier than observed if temperatures in the study were uniformly increased by 5°F and four days later if temperatures were decreased by 5°F. In a separate study with the same dataset, the author explored normalized difference vegetation index (NDVI) in relationship to the equine WNV cases in Indiana.[77] One cluster of WNV equine cases was found to have a significantly greater estimated median NDVI (0.659) for case premises than for either other case (0.571) or control (0.596) premises during the same period.

In a recent study involving wild birds caught at multiple sites in Georgia, logistic regression was used in combination with GIS to identify human and environmental variables that were important determinants of WNV distribution.[78] A database was constructed using the location of wild birds tested during May through of October of 2002, 2003, and 2004 in relation to variables on local land use classifications including

temperature, physiographic divisions, land use/landcover, and elevation, human demographic data, and statistics summarizing land cover, elevation, and climate within a 1km radius landscape around each sample point. The important variables identified were temperature, housing density, urban/suburban land use, and mountain physiographic region. It was also noted that environmental and demographic variables became less important in determining the distribution of the virus as time progressed.

The Georgia GIS study, in addition to the two Indiana GIS studies and the Chicago GIS study, strongly suggest that useful environmental risk models can be developed for WNV using GIS and remote sensing to define the ecology and epidemiology of WNV. These environmental risk models can aid WNV control and prevention programs by targeting which environmental and human factors promote the transmission of WNV.

Diagnostic Tests for West Nile Virus

Clinical signs of WNV are nonspecific and can mimic a meriod of other pathogens and toxins. Thus, an infected animal must be identified through the identification of WNV antigen or antibodies. Many tests have been developed for these purposes.

For the detection of WNV antigen, several methods have been developed. One of these methods is RT-PCR. RT-PCR is a test used for the detection of West Nile viral RNA extracted from various samples. RT-PCR works by selecting a segment of the viral gene sequence, reverse transcribes it, amplifies the cDNA, and then detects the amplified product.[1] It is a very sensitive test that can detect minute quantities of virus, but it lacks the ability to differentiate viable virus from nonviable virus. In several studies, Taqman®

RT-PCR and nested multiplex RT-PCR have detected WNV in more pools than plaque assay or VecTest™ (Medical Analysis Systems, Camarillo, CA).[79-81] Other studies have had mixed results. In terms of human WNV disease, RT-PCR has limited usefulness in human diagnostics since viral RNA cannot be detected, which is most likely due to the duration and level of viremia.[82]

Virus isolation can be carried out in a variety of cell lines, including Vero cells. Virus isolation can be used to detect previously identified or new viruses in tissues or mosquito pools. Virus isolation can vary in sensitivity from being comparable to or less sensitive as compared to RT-PCR.[80,81] Virus isolation has the ability to detect new viruses, but virus isolation is nonspecific because cytopathic effects can be due to numerous causes. Isolates must therefore be confirmed by other methods such as RT-PCR.

The VecTest is an antigen-capture assay that has been developed to test for WNV, as well as St. Louis encephalitis virus and the alphaviruses, Eastern equine encephalitis and Western equine encephalitis.[9,10] The test gives results in less than 20 minutes without use of sophisticated equipment or a biosafety level 3 lab. It has a comparable sensitivity to RT-PCR for mosquito pools.[10,83] The VecTest was originally designed for mosquito pool samples, but has since been used for birds as well. The sensitivity and specificity of the VecTest has varied. Several studies have found that the VecTest is comparable to RT-PCR when used on bird samples.[81,84] Other studies have commented on a lower sensitivity and specificity that RT-PCR. One study involving oropharyngeal swab samples from dead crows in Manitoba was found the sensitivity and specificity of the VecTest to 83.9% and 93.6%, respectively, while the sensitivity and specificity was

83.3% and 95.8%, respectively, for dead birds in Ontario.[85] In New York, the sensitivity for oral swabs ranged from 76-87%, while the specificity was 98%.[86]

WNV antibodies can be detected by a variety of techniques, but these tests are plagued by cross-reactivity between viruses. Yellow fever and dengue have cross-reacted with WNV human sera submitted for testing by HI, PRNT, and IgM ELISA tests, and this creates uncertainty in interpreting results, especially in humans.[82]

A commonly used technique for antibody detection is hemagglutination inhibition (HI). HI methods can be used to detect antibody in a wide variety of species, and does not require laboratory containment procedures if noninfectious antigen if used.[87] However, the test is laborious, tedious, and can produce nonspecific results at low antibody titers.[88] In wild birds, the extraction protocol is different than standard sucrose acetone extraction to remove nonspecific inhibitors.[82,87] In wild birds, extraction must be performed using protamine sulfate to remove nonspecific inhibitors.[87]

The plaque reduction neutralization test (PRNT) is considered the standard test for confirmation of virus-specific antibodies.[82,89,90] It detects neutralizing antibodies in either serum or cerebral spinal fluid (CSF). PRNT can be used in a wide variety of animals. PRNT is expensive to run and must be conducted within appropriate facilities since live virus is needed for the test.[88] PRNT is used as a confirmatory test for WNV antibodies by the CDC and other various laboratories in the USA. To be considered WNV positive by PRNT, there should be a fourfold higher antibody titer than other flaviviruses such as SLE virus.[89-91]

A common method for testing horses and sentinel chickens for WNV antibodies is by use of MAC-ELISA. The MAC-ELISA detects IgM antibodies in either serum or

CSF. When compared to PRNT and HI, MAC-ELISA is the test of choice for early detection of WNV antibodies.[87] The MAC-ELISA test is not useful for detecting WNV antibodies in wild birds because there is short period in which IgM antibodies can be detected, but an IgG ELISA has been developed to test wild birds for WNV antibodies.[87,92,93] The IgG ELISA demonstrates strong agreement with PRNT (kappa = 0.857) and can detect antibodies from multiple species using commercially available anti-wild bird horseradish peroxidase–conjugated antibodies.[92,93] Additionally, ELISAs have the advantage of being able to use inactivated viral antigen.[87] In terms of human testing, IgM and IgG ELISAs are commonly used for diagnostics and are usually confirmed by PRNT.[82]

Indirect enzyme immunoassay (EIA) is another method that is being used for detecting antibodies in California.[88] It was originally developed to screen wild bird sera for antibodies against WEE and SLE. In regards to WEE and SLE antibody detection in wild birds, EIA is more accurate, sensitive, and specific than HI when compared to PRNT, but EIA positive samples could not be consistently confirmed by PRNT. EIA has recently been adapted by the same developers to screen wild bird sera for WNV antibodies in California.[88,94]

Avian Serosurveys

With the introduction of WNV in the United States in 1999, many studies were launched to investigate what birds may be the most important in the transmission cycle. In New York, 430 birds from 18 species were sampled in September, 1999 from Queens and the surrounding counties.[95] Samples were tested for WNV and SLE antibodies using

PRNT. Overall, 140/430 (32.6%) birds were positive for WNV antibodies. Geese, chickens, house sparrows, and rock doves had high seroprevalences.

WNV activity was first detected in Florida in July 2001. As part of intensive surveillance efforts, free-ranging wild and captive birds were tested for antibodies to WNV in 2001.[96] Birds were sampled at 11 sites in Jefferson County. Wild free-ranging birds were caught with mist nets while captive birds were hand-caught in their enclosures. Samples from 24 species were tested by PRNT. Of the 353 birds tested, 39 (11%) were positive for WNV. Of the wild birds tested, northern cardinals, northern mockingbird, common ground-dove, common grackle, and house sparrows had the highest seroprevalence (see Appendix B for scientific names). Domestic chickens also had a high seroprevalence.

WNV was first detected in California in 2003. Free-ranging birds were caught at two wetland sites near the Salton Sea and at seven locations in Coachella Valley.[94] Birds were identified to species, sex, and age. Blood collected from each bird was tested by EIA and retested by PRNT. Testing showed 1.6% (51/3156) of birds from Coachella Valley were positive for WNV, while all 4502 free-ranging birds from Sacramento, Kern, and Los Angeles counties were negative. The highest antibody prevalences were obtained in resident columbiform and galliform species.

Free-ranging wild birds were caught at 60 sites in Illinois between 2001 and 2004 as part of the surveillance efforts for WNV.[97] Captured birds were identified by species, age, and sex if possible. Samples were tested by an epitope-blocking enzyme-linked immunosorbent assay using the monoclonal antibodies 3.1112G, 2B2, and 6B6C-1. Over the course of the study, 5,236 wild birds from 145 species were tested. Of these, 348

(6.6%) were positive for WNV antibodies. The highest seroprevalences were seen in wild turkeys, mourning doves, northern cardinals, American robins, and house sparrows (see Appendix B for scientific names). Adults had a higher seroprevalence than juvenile birds, which was attributed to antibody persistence and high site fidelity. Also, breeding birds had a higher seroprevalence than transient birds.

In Georgia, a multi-year study conducted during the summers of 2000-2004 was conducted to test 14,077 serum samples from 83 avian species for WNV.[98] Samples were tested for antibodies by PRNT against WNV and SLE. Neutralizing antibodies were detected in 869 (6.2%) of samples tested. Seroprevalence increased during the study period and was species dependent. Northern cardinals, rock pigeons, common ground doves, grey catbirds, and northern mockingbirds all had high seroprevalences (see Appendix B for scientific names). In addition, northern cardinals also had a high geometric mean titer. The authors concluded that northern cardinals have the best potential as an indicator species for surveillance of WNV in Georgia because of their extensive range, ease of capture, and high seroprevalence and antibody titers.

West Nile Virus and Experimental Infections

One of the first steps in understanding the dynamics of WNV infection in its new niche in the United States is the identification of reservoir hosts. This is important since arboviral transmission is dependent on host competency, a host's natural exposure rate to vectors, host serology, and the host's spatial and temporal availability to the vector.[51,97] One of the keys to identifying reservoir hosts is experimental infection to see which hosts can develop viremias.

Twenty-five bird species were exposed to WNV through the bite of infected *Culex tritaeniorhynchus* to evaluate their role in the transmission of the virus.[51] Of the 87 mosquito-exposed birds, 28 birds demonstrated clinical signs including lethargy, ruffled feathers, unusual posture, inability to hold head upright, and ataxia. Direct transmission of WNV between mosquito-exposed birds and cage mates were seen among ring-billed gulls, blue jays, black-billed magpies, and American crows (see Appendix B for scientific names). Blue jays, common grackles, house finch, American crow, and house sparrows were considered the most competent species based on viremia data (see Appendix B for scientific names). In addition, 15 species were evaluated for oral susceptibility to WNV. Oral WNV susceptibility was seen in great horned owls, American crows, common grackles, house finches, and house sparrows (see Appendix B for scientific names).

Domestic dogs and cats were infected by mosquito bite and evaluated as potential reservoirs for WNV.[99] Four adult, female dogs and four adult, female cats were exposed to WNV through the bite of infected *Aedes albopictus*, while an addition four cats were exposed orally through ingestion of mice. The four dogs developed low viremias for a short duration without clinical signs, while all eight cats developed higher viremias. In addition, several of the mouse infected cats had neutralizing antibodies. Three of the mosquito infected cats became lethargic and febrile after becoming infected. No attempts were made to infect mosquitoes through the bite of viremic dogs and cats; however, the authors concluded that dogs were unlikely to serve as competent amplifying hosts due to their low viral loads, while cats developed a high enough viremia to support infections of mosquitoes, but they would do so at a low efficiency.

Young adult and weanling pigs were infected with WNV through the bite of *Aedes albopictus* mosquitoes.[100] Pigs were tested for antibodies by PRNT. All of the adult pigs developed antibodies to WNV, but only one of the pigs developed a viremia. Three out of five weanling pigs developed low level viremias. None of the pigs developed clinical signs. An additional four adult pigs were orally challenged with WNV infected mice. None of these pigs developed antibodies or viremias. The results of this study suggested that pigs are not an important amplifying host for WNV.

Another experimental study in the United States looked at bats as potential amplifying hosts for WNV.[101] Big brown bats (*Eptesicus fuscus*) and Mexican free-tailed bats (*Tadarida brasiliensis*) were inoculated with WNV. In big brown bats, titers ranging from 10-180 plaque-forming units were seen beginning two days post-infection. Virus was not isolated from the Mexican free-tailed bats. Neither of the species showed clinical signs. In addition, samples were taken from 149 bats in Louisiana and tested for WNV antibodies. Only two bats were positive. The authors concluded that neither one of these species would make good amplifying hosts for WNV.

The History of West Nile Virus in Louisiana

WNV first appeared in Louisiana in 2001, when it was found in several southern Louisiana parishes, including Vermilion, Jefferson, Plaquemine, Calcasieu, and Iberia.[8] The year 2001 transmission season in Louisiana included only six WNV positive birds, one human case, and ten equine cases. The year 2002 transmission season began in January in Cameron Parish with the report of an equine case and ended with a human case in St. Tammany parish in December. In the intervening 2002 transmission season, 380 birds, 364 horses, and 329 humans tested positive for WNV. Of the 329 people

positive for WNV infection, 3 (0.9%) had an undetermined WNV syndrome, 122 (37.1%) had West Nile fever, and 204 (62%) people had the neuroinvasive form.[8] When adjusted for census population numbers, Louisiana had the second highest incidence of confirmed human WNV cases in the nation in 2002 (7.362/100,000) and ranked first in the number of mortalities due to WNV (0.559/100,000). Unlike most states, Louisiana has experienced a higher blue jay mortality rate (94%) than crow mortality rate due to WNV. In Louisiana, the main vector for WNV is *Culex quinquefasciatus* (southern house mosquito) which readily feeds on both mammals and birds.[8,101] Research has shown that *Culex quinquefasciatus* is a competent vector of WNV.[95]

In 2002, several studies were conducted in Louisiana to estimate the economic impact of WNV and to understand the epidemiology of WNV within the state. The cost of the WNV epidemic from June 2002 to February 2003 was calculated as the sum of medical costs, nonmedical costs such as productivity losses caused by illness and premature death, and the expenses incurred by public health and other government agencies.[102] Data was collected from hospitals, patient questionnaires, and public offices. The cost of WNV during this period was estimated to be $20.1 million with $10.9 million resulting from illness and $9.2 million due to the public health response.

Two studies performed in St. Tammany Parish have provided some insight into the epidemiology of WNV in Louisiana. The first study examined the seroprevalence of WNV in free-ranging mammals.[103] The study found antibodies in 13/120 (11%) of mammals tested including opossums (*Didelphis virginiana*), raccoons (*Procyon lotor*), black rats (*Rattus rattus*), hispid cotton rats (*Sigmodon hispidus*).

The other study evaluated bird species as potential amplifying hosts for WNV in St. Tammany Parish.[104] In August and October of 2002 birds were captured and bled. Samples were tested for WNV antibodies using PRNT. Antibodies were detected in 41/264 (15.5%, CI 11.7-20.4%) of resident birds in August and 40/166 (24.1%, CI 18.2-31.1%) of resident birds in October. This data along with mortality rates, host competence, and crude population estimates were then used to predict actual infection rates, population impacts, and the potential of several species of birds found in Louisiana as amplifying hosts. Northern cardinals and house sparrows were identified as major the major amplifiers of WNV, with blue jays and northern mockingbirds playing a significant role.

In the year 2003, a decrease in the number of WNV positive humans and horses were reported in Louisiana. Only 101 (82.8%) WNV human neuroinvasive and 21 (17.2%) WNV fever or undetermined cases were recorded.[8] There were only 36 equine cases of WNV, but there were 13 other animals, including 7 alligators, that were WNV positive. Other WNV tests showed positive results in 390 dead birds, 387 positive sentinel chickens, and 201 positive mosquito pools.

In 2004, the number of WNV positive mosquito pools increased to 909 due to more sensitive test methods.[8] The number of WNV human cases decreased slightly to 109 total cases with 84 (77.1%) being neuroinvasive. The number of WNV infected horses increased to 70 while the number of positive sentinel chickens decreased to 157.

In 2005, the number of WNV positive mosquito pools increased even more to 1441 positive pools, with 1395 (96.8%) of those pools being reported as *Culex quinquefasciatus*.[8] The total number of human cases rose slightly to 187 cases with 117

(62.6%) of those being reported as neuroinvasive. WNV equine cases decreased dramatically to only 16 reported cases, while WNV positive sentinel chickens rose to 177 birds. The decreased prevalence in horses was likely due to widespread use of efficacious WNV vaccines. As these figures indicate, WNV has become endemic in Louisiana and will likely continue to present a risk to humans, birds, and horses. Even though several studies have yielded some information about the epidemiology of WNV in Louisiana, further study is needed to gain a wider understanding of the epidemiology and ecology of WNV to prevent disease and decrease the impact of WNV throughout the state.

Study Objectives and Hypotheses

WNV has had a major impact on Louisiana as a cause of morbidity and mortality in humans and animals. The goal of the studies presented here is to expand our knowledge base regarding the epidemiology and ecology of WNV in Louisiana. This will be accomplished using several methods that identify key components of the WNV transmission and life cycles in Louisiana. First, previous research has suggested environmental and human factors influence WNV transmission. A GIS database will be constructed using WNV positive dead birds and WNV human cases in conjunction with environmental datalayers to identify factors that favor WNV transmission. It is hypothesized that thermal and hydrologic indicators will be among the factors identified as promoting WNV transmission since these factors contribute significantly to the life cycle of WNV vectors.

Second, an avian serosurvey will be conducted in East Baton Rouge Parish at multiple locations to identify key species involved with WNV amplification,

transmission, and spillover to the human population. It is hypothesized that several bird species will be identified as important hosts for WNV in Louisiana by having a higher seroprevalence than the overall seroprevalence. It is also hypothesized that adult wild birds will have a higher seroprevalence than juvenile wild birds since antibodies can persist for an unspecified length of time. Additionally, several locations should emerge as hot spots conducive to WNV transmission.

Finally, birds are the main reservoirs for WNV, but other animals such as alligators have also been implicated as reservoirs since they have been shown to amplify WNV to a level high enough to infect mosquitoes and humans. The third study evaluates the Mediterranean House Gecko (*Hemidactylus turcicus*) as a potential reservoir for WNV. Geckos are insectivorous, so it is speculated they can become infected through the ingestion of a WNV infected mosquito. It is hypothesized geckos can become infected with WNV and that they can act as a reservoir for WNV.

DEVELOPMENT OF A PREDICTIVE MODEL FOR WEST NILE VIRUS IN LOUISIANA USING GEOGRAPHIC INFORMATION SYSTEMS AND REMOTE SENSING

Introduction

Arboviruses, as their name implies, are dependent on an insect vector for both transmission and development. Arbovirus' dependency on insect vectors also makes them susceptible to the same environmental influences that can affect their vector. Many studies have looked at the effect that temperature and other environmental factors have on the development of viruses such as Dengue, St. Louis encephalitis, Western equine encephalitis, and Eastern equine encephalitis.[66-69] Specifically, increased temperature has been shown to shorten the extrinsic incubation time of the virus in the mosquito host. It is proposed that the unique environmental preferences and limits of tolerance of different arbovirus-vector systems may be used to develop geospatial risk models for predicting when and where the disease will occur.

Since the introduction of WNV into the United States, several attempts have been made to develop models that can predict the temporal and spatial distribution patterns of WNV. Most of these models are based on surveillance data gathered on WNV. Simple models in the northeastern USA have only used dead bird sightings and reports to make predictive models.[61-64] More comprehensive models have been constructed based on the influence of environmental determinants on WNV transmission using geographic information systems (GIS) and remote sensing (RS). One group of researchers used Advanced Very High Resolution Radiometer (AVHRR) images in conjunction with dead bird records to predict the distribution of WNV in North America.[106] State level studies in Indiana by Ward in 2005 evaluated the relationship of temperature and vegetation

variables with equine WNV cases to identify potential areas of elevated risk.[76,77] In Georgia, several environmental variables including temperature and suburban/urban landuse were identified as important.[78] The Georgia study also identified a non-environmental variable, housing density, as an important variable. On the local urban level, a study of WNV in Chicago revealed environmental variables such as vegetation and geology, and non-environmental factors such as mosquito abatement, age, and income, as important determinants of human WNV disease.[74]

The intent of the present work is to show that geospatial analysis, climate and satellite surveillance tools can be used, with intrinsic environmental determinants of the vector-viral-vertebrate development cycle, to develop a predictive model for WNV spatial-temporal risk that can be used to guide control programs in Louisiana. Previous studies on WNV in *Culex* spp. have shown that temperature plays an important role in virus replication and transmission.[69,70] Specifically, it is hypothesized that hydrologic and thermal factors will be the most important environmental variables in Louisiana since climate greatly influences both the life cycle of the mosquito and the extrinsic incubation period of the virus.

This work will be accomplished by analysis of WNV positive dead bird and human data from Louisiana in 2002 and 2003 in relation to map-datalayers on environmental features, satellite imagery, and climate grid data within a GIS project. In addition, several non-environmental factors, such as presence or absence of abatement districts and population data, will be evaluated in the development of a WNV model for Louisiana.

Experimental Methods and Design

A WNV predictive model was developed using the GIS programs ArcView 3.3 and ArcGIS 8.3 (ESRI, Redlands, CA) and the raster image processing software Erdas Imagine (Leica, Atlanta, Georgia). The model developed in this study explores the relationships between environmental risk factors including temperature and vegetation and WNV positive data related to humans and dead birds from 2002 and 2003. Environmental data that was evaluated within the GIS included both image data and vector map data (points, lines, polygons). Environmental image data will include land surface temperature (LST) and normalized difference vegetation index (NDVI) from the MODIS earth observing satellite system as well as maximum temperature, minimum temperature, and precipitation from a NASA climatology database. Vector map data was mostly derived from the Louisiana GIS CD: A Digital Map of the State.

WNV positive human case databases were obtained from the Louisiana Department of Health and Hospitals Office of Public Health (OPH). WNV positive dead bird data was acquired from the Louisiana Veterinary Medical Diagnostic Laboratory (LVMDL) at the Louisiana State University School of Veterinary Medicine (LSU-SVM). The human and dead bird information was entered into an Excel table (Microsoft, Redmond, Washington) and exported as dbase IV files in Excel so they could be manipulated in ArcView 3.3. Data layers or shapefiles were created by geocoding addresses associated with human and bird data in ArcView 3.3 using the ArcView Streetmap USA extension. Geocoding involves matching an address to a latitude and longitude coordinate. In some instances, zip codes were used instead of addresses due to incomplete or inaccurate records. The resulting output was a point shapefile. After being

geocoded, the ArcView script, Polycent, was used to add latitude and longitude coordinates of every data point. Overall, 306/380 (80.5%) dead birds and 306/329 (93%) humans were geocoded for 2002. In 2003, 367/392 (93.6%) dead birds and 122/122 (100%) human cases were geocoded.

Once the data was geocoded, a Julian date code was assigned to each WNV data point. A Julian date corresponds to the number of days that have elapsed since January 1. The Julian date code assigned to each point corresponded with the presumed date of infection since the model was meant to explore factors that influence the transmission of WNV. In humans, it was estimated that infection occurs approximately three days prior to the onset of clinical signs.[32,107,108] In birds, it was estimated that infection occurs five days prior to finding a dead bird.[108] The five-day period also assumed that dead birds were not found immediately after death. Using a Julian date code enables the grouping of human and dead bird data into temporal cohorts.

Moderate Resolution Imaging Spectroradiometer Imagery

Images from the Moderate Resolution Imaging Spectroradiometer (MODIS) sensor aboard the Terra earth orbiting satellite were obtained from Earth Resources Observation and Science (EROS) Land Processes Distributed Active Archive Center (LP DAAC, Sioux Falls, South Dakota). The two products that were used were LST and NDVI. LST images were acquired as 8-day composites at one kilometer resolution, while NDVI images were acquired as 16-day composites at 250 meter resolution.

MOD11 (LST) and MOD13 (NDVI) MODIS images were imported from HDF-EOS file format into ERDAS Imagine file format. For MOD11 images, LST_Day_1km and LST_Night_1km were the layers imported while 250m 16 day Normalized

Difference Vegetation Index (NDVI) was the layer imported from the MOD13 file. After the images were imported, the images were mosaicked. This was accomplished by adding images for the southeastern United States in the mosaic window in Erdas Imagine. The images, and all other map datalayers in the GIS, were georectified to the geographic latitude-longitude, WGS 84 spheroid and datum using the geometric correction tool in ERDAS Imagine. The projection of images was originally defined as integerized sinusoidal, WGS 84. Projection type was selected as geographic latitude and longitude while spheroid and datum were chosen as WGS 84. RMS error did not exceed one. An area of interest (AOI) file was created in ERDAS Imagine to clip out only the area within the state of Louisiana. Finally, LST images were multiplied by 0.02 to transform satellite LST values to temperature in degrees Kelvin. NDVI images were multiplied by 0.0001 to normalize the vegetation index values between -0.3 – 1.0.

Thermal images were further processed in ArcGIS to eliminate cloud cover by assigning null values to zones that fell below a temperature threshold of 263K (-10°C). This cloud mask threshold was used because it was the lowest temperature that had been validated according to MODIS LST Products Users' Guide by the Institute for Computational Earth System Science at the University of California-Santa Barbara.[109] NDVI images were processed further in ArcGIS to exclude values defined as no data (values ≤ -0.3) by the Terrestrial Biophysics and Remote Sensing Lab at the University of Arizona, the creators of the MODIS NDVI product (Figure 1).[110]

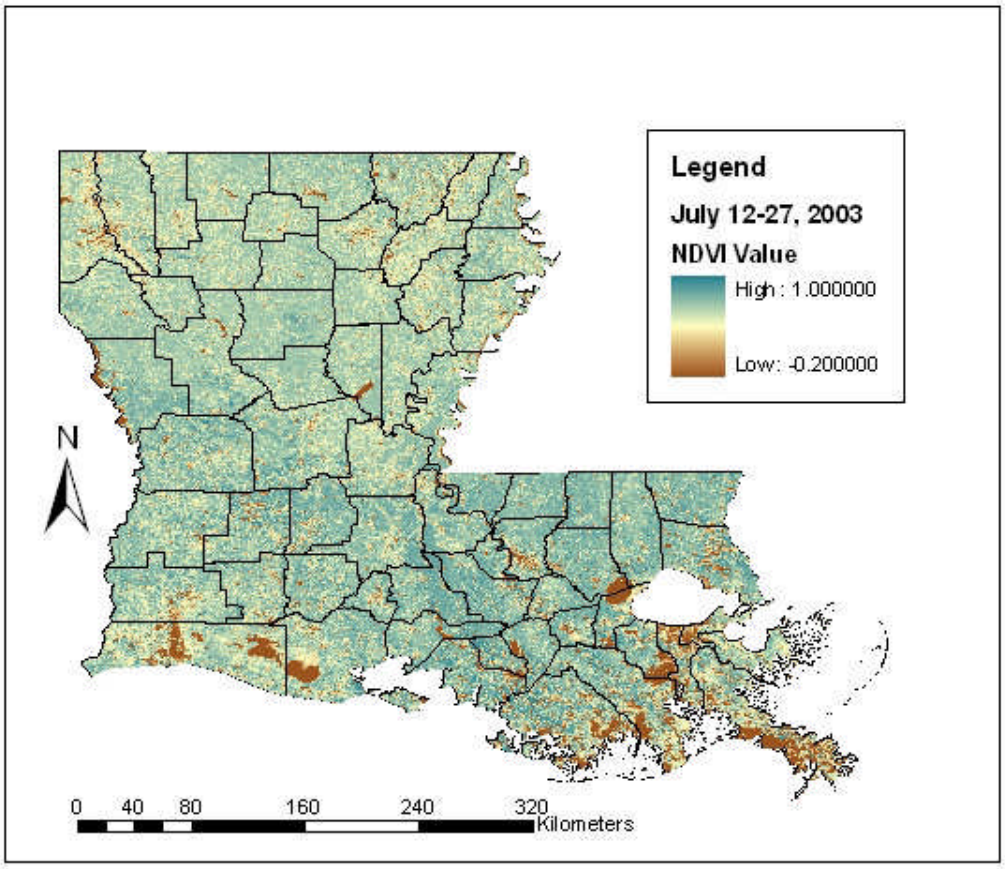

Figure 1: MODIS normalized difference vegetation index (NDVI) image for the 16-day period of July 12-27, 2003. Image processed using ERDAS Imagine and ArcGIS.

The dead bird and human shapefiles were divided into 8-day cohorts based on Julian dates assigned to each point. The 8-day cohorts corresponded to MODIS composite dates for each LST image. Since NDVI composites were based on 16-day composites, NDVI images were used for the two cohort groups that corresponded to the 16-day period. LST and NDVI values from the images were obtained by creating 2.5 kilometer buffers with the Spatial Analyst extension in ArcGIS around human and dead bird data points. Buffers were joined to the point shapefile for the humans and dead birds and extractions were performed in ArcGIS to obtain the mean, range, standard deviation, minimum, and maximum for the raster layers NDVI, day LST, night LST, and day night

LST differences. These values were exported into a dbase IV table using ArcGIS for further analysis.

NASA Climate Grid

Gridded climate data files for maximum temperature (Tmax), minimum temperature (Tmin), and precipitation were obtained from NASA. In contrast to the MODIS images, the temperature information imbedded in the grids reflected air temperature and not LST. The grids also had a courser resolution, 8km, compared to the MODIS images. The NASA datasets were derived from meteorological station data taken from a variety of networks including local statewide networks and is designed to be used in ecological forecasting. The datasets were then projected onto the land surface and interpolated using the Surface Observation and Gridding Surface (SOGS). SOGS is largely based on the Daymet algorithms.[111,112]

Files were obtained from NASA in generic binary data format. These files were imported into ERDAS Imagine. The files were imported in data format BSQ, data type IEEE 32 bit float. The column value was defined as 76 while the row value was defined at 64. Once the raster images were imported, the projections were defined. This was accomplished by selecting 'information' in the ERDAS Imagine toolbar. The Upper Left X was defined as 510000 while the Upper Left Y was defined at -122400. Both X and Y pixel sizes were input as 8000 meters while the projection was listed as Lambert Azimuthal Equal-Area. The map model was then changed to read sphere of radius 6370997m, undefined datum, 100 W as the longitude of center of projection, and 45 N as the latitude of center of projection. Eight-day composites based on the MODIS LST dates were constructed using ERDAS Imagine (Figure 2).

Extractions were performed using the 8-day human and dead bird cohorts. Extractions were performed manually using the information tool in ArcGIS without the use of buffers. The information tool was used to identify precipitation, Tmax, and Tmin values for each point. These values were then typed in Excel.

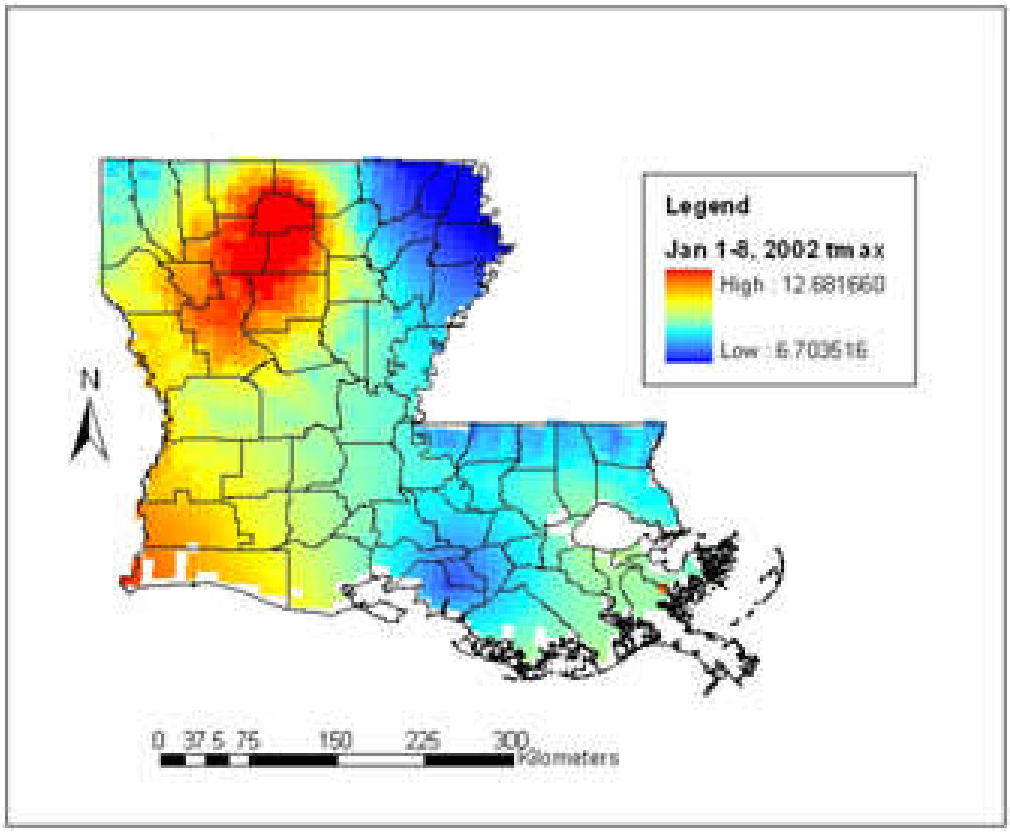

Figure 2: NASA climate grid depicting maximum temperature (°C) for the 8-day period of January 1-8, 2002. Image was processed using ERDAS Imagine and ArcGIS.

The Louisiana GIS CD: A Digital Map of the State

The Louisiana GIS CD: A Digital Map of the State (Louisiana Oil Spill Coordinator's Office, Baton Rouge, Louisiana) database contains many GIS layers for the state. The GIS layers from this database used in the WNV predictive model analysis

were aquifers, flood zones, geology, soils, wildlife refuges, wildlife management areas, water bodies, and streams. A parish shapefile was also used so that a parish population extraction could be performed. The population for each parish was obtained from 2000 Bureau of Census statistics, entered into an Excel table, exported as a dbase IV file, and then joined to the parish shapefile.

The recharge potential of the Louisiana aquifer layer was created by the Louisiana Department of Environmental Quality (LDEQ) and published in 1999. The coordinate system of the layer was geographic latitude/longitude, projection North American Datum (NAD) 83, ellipsoid geodetic reference system 1980 (GSR 1980). This is a polygon dataset depicting the boundaries of aquifer systems in the state of Louisiana and adjacent areas of Texas, Arkansas and a portion of Mississippi. These aquifers have been classified as to recharge potential from none to high. The data set was developed by digitizing maps of the State Aquifer Recharge Map and Atlas Plates.

In 1998, the United States Geological Survey (USGS), Biological Research Division's National Wetland Research Center published the digital overlay of the geologic map of Louisiana. The coordinate system of the geologic layer was geographic latitude/longitude, NAD83, ellipsoid GSR1980. Data, in the form of a digitized overlay of geologic areas, was collected from the Geologic Map of Louisiana. Polygons were digitized, in Arcview 3.0, on the computer screen. Polygons were subsequently edited and assigned attributes within the Arcview environment. The shapefiles were then converted to ARC/INFO coverages and cleaned.

The Federal Emergency Management Agency (FEMA) published the quality level 3 (Q3) flood data map in 1995. The coordinate system for the data set was geographic

latitude/longitude, NAD 27, ellipsoid Clarke 1866. The Q3 flood data are countywide vector files derived from FEMA hardcopy flood insurance rate maps (FIRMs), new engineering data, and digital base mapping. The hardcopy FIRMs consist of FEMA hydrography, flood hazard zones, base flood elevations, cross-section locations, and elevation reference marks.

In 1999, the Louisiana Oil Spill Coordinator's Office (LOSCO) published the major waters of Louisiana. The coordinate system for the major waters data layer was the same as the aquifer dataset. The Louisiana major water dataset is a region dataset that contains areas that are major water features - lakes and streams wide enough to be represented as areal features on large scale maps (Figure 3). The dataset was derived from an ESRI data set created from Dynamap 2000 v7.3 (Geographic Technology Dynamap, Inc., Lebanon, New Hampshire) release data. Louisiana features were extracted from MJWATER using the spatial overlay function of ArcView.

LOSCO published the Louisiana rivers and streams GIS layer in 1999 with the same projection as major waters, aquifers, and geology. This is a line dataset that depicts most of the major and minor streams, rivers and other waterways in the state of Louisiana (Figure 3). It was derived from an ESRI data set created by Geographic Data Technology, Inc. Dynamap 2000 v7.3 data that was in turn derived from the Bureau of the Census TIGER/Line files.

LOSCO published the National Wildlife Refuges in Louisiana from the Nature Conservancy Source Data in 1998. The projection for the dataset is the same as Louisiana aquifer dataset. The GIS layer represents the polygon boundaries of the National Wildlife Refuges in the Louisiana that are administered and owned by the

federal government (Figure 3). The dataset was digitized by The Nature Conservancy from U.S. Fish & Wildlife Service FOR STATUS ONLY maps.

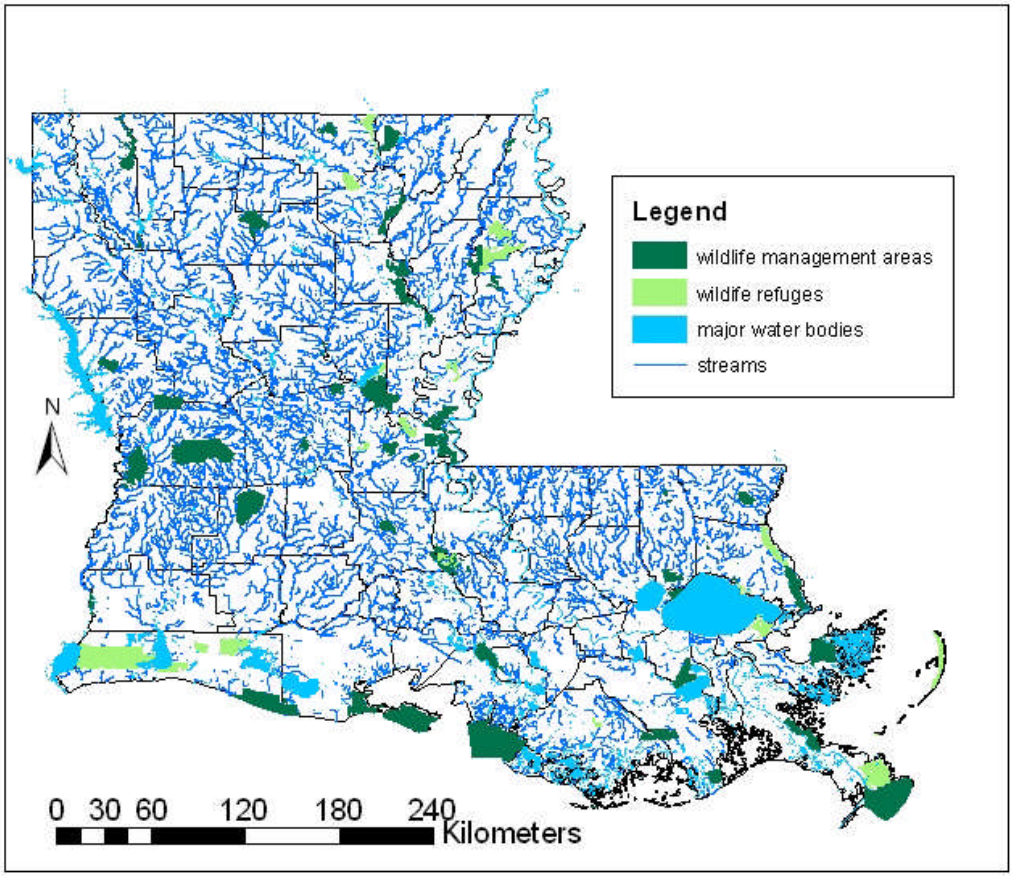

Figure 3: Map showing the shapefiles wildlife management areas, wildlife refuges, streams, and major water bodies within Louisiana. The shapefiles are part of the database on The Louisiana GIS CD: A Digital Map of the State compiled by the Louisiana Oil Spill Coordinator's Office.

The wildlife management area dataset from the Louisiana Oil Spill database used for this project was published in 1998. The dataset was in the projection already defined for other GIS layers on The Louisiana GIS CD: A Digital Map of the State. These data represent the polygon boundaries of Wildlife Management Areas in Louisiana. Wildlife Management Areas are administered by the states, but may be privately owned (Figure

3). This is a subset of a larger data set produced for the Southcentral United States by the Nature Conservancy (TNC). Wildlife Management Areas were digitized by TNC from maps published by the Louisiana Department of Wildlife and Fisheries.

In 1998, the U.S. Geological Survey's National Wetlands Research Center published the general soil associations map. In 1999, Louisiana State University projected the GIS layer to decimal degrees, NAD 83, and ellipsoid Geodetic Reference 80 to match the other layers on The Louisiana GIS CD: A Digital Map of the State. This data set contains vector line map information. The vector data contain selected base categories of geographic features, and characteristics of these features, in digital form. The information was collected by clipping the existing STATSGO soils map to the State of Louisiana boundary. The data set used the existing STATSGO soil maps as a data development resource. The classified data was derived from actual field identification and sampling performed by the United States Department of Agriculture and the Soil Conservation Service.

These shapefiles were added to the ArcGIS 8.3 resource database for analysis within a GIS. Point to polygon extractions were performed using the human and dead bird point data shapefiles. A point to polygon extraction was used for two reasons. First, most of the data contained in these shapefiles was categorical data meaning only a non-metric value could be derived. Second, wildlife refuges, wildlife management areas, water bodies, and streams are not continuous throughout the state, so a point to polygon extraction had to be used to acquire distances from a particular place that was part of the shapefile. After data from these shapefiles was acquired, they were exported as dbase IV files.

EPA website

Other datalayers such as forest density, forest type, and land use/land cover were downloaded through the Environmental Protection Agency (EPA) website (http://www.epa.gov/airmarkt/cmap/data/category3.html). The forest density GIS layer was published in 1992 by U.S. Department of Agriculture, Forest Service, Southern Forest Experiment Station, Forest Inventory and Analysis (SO-FIA). The dataset is an ArcInfo grid of the conterminous United States (48 states), projected in Albers Equal-Area meters, GRS1980, NAD83. The 1km grid indicates percent forest cover from 0-100%. The dataset was produced in support of the Forest and Rangeland Renewable Resources Planning Act (RPA) 1993 Assessment Update program. The data were created to provide information on current forest and rangeland conditions. The data are based on Advanced Very High Resolution Radiometer (AVHRR) data from the National Oceanic and Atmospheric Administration (NOAA) satellites.

In 1992, U.S. Department of Agriculture, Forest Service, SO-FIA published the GIS data layer forest type. The projection for this layer is the same as the one for forest density. This dataset contains forest types grouped into 22 general categories. The data were produced in 1991 by the U.S. Department of Agriculture, Forest Service, Southern Forest Experiment Station, SO-FIA research unit, in support of the Forest and Rangeland Renewable Resources Planning Act (RPA) 1993 Assessment Update program. The purpose of the data was to provide information on current forest and rangeland conditions. This layer is also based on Advanced Very High Resolution Radiometer (AVHRR) data from NOAA satellites.

In 1990, the USGS EROS Data Center and the Advanced Land Management Information Technologies University of Nebraska – Lincoln created a land cover GIS dataset for the contiguous 48 states. It has a pixel size of approximately 1km, and is projected as Albers Equal-Area, GRS1980, NAD83. The original source data used for classification of seasonally distinct land cover regions were NOAA Advanced Very High Resolution Radiometer (AVHRR) High Resolution Picture Transmission (HRPT) satellite imagery. The preliminary classification of land cover characteristics in the 1990 prototype study was derived from a time-series of 8 AVHRR 28-day maximum NDVI composite images spanning the 1990 growing season (March 16-October 25). The AVHRR time-series of eight 28-day maximum NDVI composites for March-October 1990 were initially stratified into vegetated and non-vegetated regions using a maximum NDVI image representing this 8-month period. Biweekly AVHRR composites were also used in the process of land cover class refinement. An unsupervised classification using a clustering algorithm (ISOCLASS) and minimum-distance-to-mean classifier was used to define 70 spectral-temporal (seasonally distinct) classes within the vegetated stratum. Ancillary data on ecosystems, topography, and climate were used to sort and identify specific cover types, thus refining the 70 initial classes into 159 seasonally distinct land cover regions.

These files were downloaded as ESRI interchange files and were converted to shapefiles using ArcGIS 8.3. The shapefiles were then clipped to include only the area within the boundary of Louisiana using Erdas Imagine and extractions were performed as described earlier for shapefiles on The Louisiana GIS CD: A Digital Map of the State.

Louisiana Gap Analysis Program Data

Louisiana Gap Analysis Program (LA GAP) data was obtained from the Louisiana Atlas website's (http://atlas.lsu.edu/) link to the LA GAP shapefiles. In 1998, the LA GAP and the U.S. Geological Survey (USGS) Biological Research Division's National Wetlands Research Center published the land cover classification for the LA GAP as part of the National GAP project. The dataset was projected as universal transverse mercator (UTM), NAD27, Clarke 1866. This data set consists of digital data describing the land use/land cover (mainly vegetation, but including water and urban environments) for the State of Louisiana at 1:100,000 scale. The map was compiled using Landsat 5 Thematic Mapper satellite imagery, botanical surveys, color infrared aerial photography, and existing coastal Louisiana habitat maps produced by the U.S. Fish and Wildlife Service's National Wetlands Inventory. Band 3 (visible red), Band 4 (near infrared), and Band 5 (mid-infrared) were the best band combinations for classifying the data. Attribute fields describe the different land cover types that occur within a polygon or that are associated with each pixel.

The LA GAP shapefile required extensive processing. It was imported as an ERDAS Imagine file and converted from a raster layer to a polygon using ArcGIS 8.3. After conversion, the dbase IV file associated with the resulting polygon was joined to a dbase IV file with the descriptive names of the land cover and land use types. The product of this join was exported as a polygon shapefile called LA GAP. Extractions were performed as previous described for the shapefiles from the Louisiana Oil Spill database.

Master Database Construction

Following completion of all extractions, a master database was constructed that corresponded to human and bird databases for both 2002 and 2003. Both databases contained parish, presence of absence of mosquito abatement district (abatement), x- and y-coordinates, aquifer, flood zone, forest type, forest density, land use/land cover, geology, LA GAP data on land use, soil type, soil hydrology, and measurements of distance of case data points from streams (distance_streams), water bodies (distance_waterbodies), wildlife management areas (distance_areas) and wildlife refuges (distance_refuges). Also included were the climate values for precipitation, Tmax, and Tmin from the NASA datasets. The MODIS satellite images produced minimum (min), maximum (max), mean, standard deviation (std), and range values for NDVI, day, night, and difference (diff) extractions from buffers centered on human and bird data points. The human master database also contained date of onset of clinical signs (onset) and human population while the bird database contained species and date found (Table 1).

Upon completion of the entry of all extracted data into the master database, the data was manipulated so it could be processed for statistics by SAS® 9.1.3 (SAS Institute Inc., Cary, North Carolina). First, a period was inserted into every missing data value so SAS would recognize the value was missing. Forest density was changed from a percent to a category. For instance, 0-10% was changed to one, 11-20% was changed to two, and so on. After forest density was changed, the 159 categories for the USGS EROS land use/land cover data were truncated to the first vegetation type listed for each category since three or more vegetation types were listed for each category. Next, loess was dropped from several categories listed for geology to reduce the number of categories.

Finally, the LA GAP output was reduced to several categories. Reduction in some of the categories for the variable was necessary for convergence to occur.

Table 1: Human and Dead Bird Variables Derived from GIS and Remote Sensing Data Included in Master Database for Statistical Analysis

NDVI_mean	NDVI_min	NDVI_max	NDVI_range	NDVI_std
Day_mean	Day_min	Day_max	Day_range	Day_std
Night_mean	Night_min	Night_max	Night_range	Night_std
Diff_mean	Diff_min	Diff_max	Diff_range	Diff_std
Tmax	Tmin	Precipitation	Aquifers¨	Flood zones¨
Distance_refuges	Distance_areas	Distance_waterbodies	Distance_streams	Geology¨
Forest Density¨	Forest Type¨	Land Use/Land Cover¨	LA GAP¨	Coordinates
Soil Hydrology¨	Soil Type¨	Parish¨	Date of Onset*	Population*
Species+¨	Date Found+	Mosquito Abatement¨		

NDVI, normalized difference vegetation index (MODIS); Day, day temperature (MODIS); Night, night temperature (MODIS); Diff, day-night temperature difference; mean, mean statistical value; min, minimum statistical value; max; maximum statistical value; range, range of statistical values; std, standard deviation of statistical value; Tmax, maximum temperature (NASA); Tmin, minimum temperature (NASA); Precipitation (NASA); Aquifers (LOSCO); Flood Zones (LOSCO); Distance_refuges, distance from wildlife refuges (LOSCO); Distance_areas, distance from wildlife management areas (LOSCO); Distance_waterbodies, distance from waterbodies (LOSCO); Distance_streams, distance from streams (LOSCO); Geology (LOSCO), Forest Density (EPA); Forest Type (EPA); Land Use/Land Cover (EPA); LA GAP, Louisiana Gap Analysis Program; Coordinates, x- and y-coordinates; Soil Hydrology (LOSCO); Soil Type (LOSCO); Date of onset, onset of clinical signs in human patients; Date found, date dead birds found; Mosquito Abatement, presence or absence of mosquito abatement district; * Human Only; + Bird Only; ¨ Categorical Variable

The prevalence of WNV for humans and birds was then calculated. The human prevalence was based on how many people were estimated to be infected with WNV. This was accomplished by using the CDC estimate for neuroinvasive WNV cases, which suggests there are approximately 150 people infected with WNV, and for every WN Fever case, there are approximately five people infected with WNV. The number of WNV neuroinvasive cases or WN Fever cases in instances where WNV neuroinvasive cases were lacking were added for every parish and then multiplied by the appropriate factor. This product was then divided by the parish population. The end result was used

as an estimate for the prevalence of WNV by parish. Bird estimates were obtained by dividing the number of positive birds by the number tested for each parish.

Statistical Analysis

The linear regression analysis included 31 variables for the bird datasets and 32 variables for the human datasets. This was accomplished by running PROC CORR in SAS 9.1.3. PROC CORR calculated a Pearson's and a Spearman correlation coefficient. A multiple linear step-wise regression model was run using the PROC REG function. Linear regression models were run separately for 2002 and 2003 bird data sets and human infection data sets. In addition, bird and human datasets were combined to form one bird dataset and one human dataset to examine what the addition of the variable 'year' would influence outcome of the analysis.

The best fitting models were those that had high R-square values (the coefficient of determination which measures the relative strength of corresponding regression), low Mallows C(p) values (a reflection of the number of parameters), and significant individual p-values for the variables. The best fitting models for each year and combined years for birds and humans were then examined for multicollinearity or correlation between the independent variables since many of the variables are related to each other. Variance of inflation (VIF) values were calculated for each of the best fitting models to determine if multicollinearity existed. VIF values less than ten indicated variables did not demonstrate multicollinearity. PROC GLM Least Squares (LS) MEANS analysis was used to detect significant differences in the means of the metric variables.

Categorical variables, except for the presence or absence of abatement district, were not included in the linear regression analysis since many categories existed for each

variable which would affect convergence. In addition, the level of measurement would be reduced if logistic regression was performed. For categorical variable analysis, chi-square tests were performed using Excel to compare the observed frequency for each category to its expected frequency. Expected frequencies for each category were computed by calculating the percent area for each category in ArcGIS and multiplying that percent by the total number of points for that variable. Individual chi-square values for each category were compared to the critical value of 3.84 which corresponds to a p-value of 0.05 for one way chi-square tests.

Results

The best bird model was a combined 2002 and 2003 model with nine variables including year (Table 2). This model had an R-square of 0.5654, Mallows C(p) value of 7.0505, and all individual p-values were less than 0.0663. The R-square indicates that over 56% of the variation seen in the percent of birds testing positive was due to these nine variables. All VIF values were less than ten, indicating multicollinearity was not a major confounding problem in the resulting model.

The best fitting 2002 bird model was a five variable model that had an R-square of 0.2481 while the best fitting 2003 bird model was a six variable model that had an R-square of 0.4086. Both of these R-square values were lower than the R-square reported for the combined model. All three best fitting bird models had the variables x-coordinate and mosquito abatement district in common.

The best model based on human prevalence data and the overall best model was a 13 variable developed from the 2003 human infection data (Table 3). The model had an R-square of 0.7448, indicating that over 74% of the variation in the human prevalence for

2003 could be explained by the variables within the model. The Mallows C(p) value was 14.1397 and all individual p-values for the variables (p-value ≤ 0.0778) were accepted as significant. The highest VIF for the model was 8.18338 indicating that multicollinearity was not a major influence on the outcome of the model.

Table 2: Variables Included in the Combined Year (2002 and 2003) WNV Dead Bird Regression Model with an R-Square of 0.5654

Variable	Parameter Estimate	Partial F Statistic	P-Value	Variance of Inflation
Intercept	697.86696	321.16	<.0001	0.00000
Distance_refuge	0.07042	4.68	0.031	1.77217
Precipitation	0.00555	4.58	0.0329	1.20245
Tmax	0.02175	9.78	0.0019	5.86959
Tmin	-0.01476	7.86	0.0053	5.22956
NDVI_min	0.12044	3.39	0.0663	1.25396
X-coordinate	0.03598	11.91	0.0006	2.47934
Y-coordinate	0.01925	13.24	0.0003	1.85523
Abatement	-0.0597	6.57	0.0107	1.10891
Year	-0.34714	315.97	<.0001	1.48272

Distance_refuge, distance from wildlife refuges (LOSCO); Precipitation (NASA); Tmax, maximum temperature (NASA); Tmin, minimum temperature (NASA); NDVI_min, minimum normalized difference vegetation index (MODIS); X,Y-coordinate, coordinate of data point; Abatement, presence or absence of mosquito abatement; Year, year of data

The best 2002 human model was a six variable model that had an R-square of 0.3153 while the combined human data for 2002 and 2003 model was a six variable model with an R-square of 0.1474. Despite having substantially lower R-squares than the best fitting human model, these models shared many variables in common with the 2003 human model.

Separate analyses were also performed on only environmental variables for 2002 and 2003 human and bird models. Analysis showed a 2003 human model with nine variables, all of which were significant, had an R-square value of 0.5368 indicating that

environmental variables accounted for over 53% of the variation in the model. Table 4 shows the variables included in this model.

Table 3: Variables Included in the 2003 WNV Human Regression Model with an R-Square of 0.7448

Variable	Parameter Estimate	Partial F Statistic	P-Value	Variance of Inflation
Intercept	0.89973	3.19	0.0778	0.00000
Distance_refuge	0.00495	5.52	0.0213	5.26578
Distance_streams	0.17606	16.68	0.0001	1.18127
NDVI_mean	0.02385	10.08	0.0021	1.87661
Night_mean	0.00057	4.91	0.0296	1.53583
Precipitation	0.00064	10.20	0.0020	1.47421
Population	1.66757E-08	4.18	0.0442	3.34486
Tmin	-0.00058	3.51	0.0647	7.86940
Day_std	0.00117	6.20	0.0149	3.17136
Diff_min	0.00080	18.71	<.0001	4.69377
X-coordinate	0.00322	8.69	0.0042	8.81338
Y-coordinate	0.00460	15.33	0.0002	5.46294
Abatement	0.00789	24.73	<.0001	2.18719
Onset	-0.00006	4.27	0.0420	5.01025

Distance_refuge, distance from wildlife refuge (LOSCO); Distance_streams, distance from streams (LOSCO); NDVI_mean, mean normalized difference vegetation index (MODIS); Night_mean, mean night temperature (MODIS); Precipitation (NASA); Tmin, minimum temperature (NASA); Day_std, standard deviation for day temperature (MODIS); Diff_min, minimum day-night temperature difference (MODIS); X,Y-coordinate, coordinate of data point; Abatement, presence or absence of mosquito abatement; Onset, date of onset of clinical disease

The chi-square tests performed on the categorical variables showed consistent results for both models developed using dead birds and human prevalence data. For instance, the observed values for the Louisiana GAP category ‘vegetated urban’ were consistently higher and significantly different from the expected values for dead birds and human data during 2002 and 2003 (Table 4). The Louisiana GAP category ‘non-

vegetated urban' was also significantly different for every group except for the 2003 human data.

Table 4: Variables Included in 2003 Human WNV Environment Only Regression Model with an R-Square of 0.5386

Variable	Parameter Estimate	Partial F Statistic	P-Value	Variance of Inflation
Intercept	-0.44025	-4.17	<0.0001	0
Dist_refuge	0.00622	3.88	0.0002	1.76246
Dist_streams	0.21815	4.07	0.0001	1.05819
Tmax	-0.00104	-2.19	0.0313	7.07953
Day_max	0.00108	3.71	0.0004	4.91008
Diff_range	-0.00064	-2.79	0.0065	2.8103
NDVI_mean	0.01769	2.17	0.0331	1.2831
Night_mean	0.00132	3.00	0.0036	9.45724
Night_min	-0.00086	-2.48	0.015	7.48322
Precipitation	0.00071	2.62	0.0105	1.54704

Dist_refuge, distance from refuges (LOSCO); Dist_streams, distance from streams (LOSCO); Tmax, maximum temperature (NASA); Day_max, maximum day temperature (MODIS); Diff_range, range in temperature day-night temperature difference (MODIS); NDVI_mean, mean normalized difference vegetation index; Night_mean, mean night temperature (MODIS); Night_min, minimum night temperature; Precipitation (NASA)

Table 5: One Sample Chi-Square Test for LA GAP Category Vegetated Urban for 2002 and 2003 WNV Positive Dead Birds and WNV Human Cases

Dataset	% Area	Total Points	Expected Points	Observed Points	Chi-Square Test Statistic
2002 Bird	0.024153	291	7.028523	164	3505.721557*
2003 Bird	0.024153	347	8.381091	193	4066.790536*
2002 Human	0.024153	303	7.318359	176	3887.961223*
2003 Human	0.024153	121	2.922513	54	892.6939515*

*p-value ≤ 0.001; Chi-square test = $(\text{Observed-Expected})^2/\text{Expected}$; Vegetated Urban category taken from Louisiana Gap Analysis Program dataset

Other significantly different categories include 'natural levees' and 'prairie terraces' which are categories within the geology variable. Dead birds and human cases

also had higher than expected frequencies for the forest type category ‘non-forest’ and the ‘forest density’ categories of less than or equal to 30%.

Discussion

The best fitting model developed by statistical analysis was the 2003 WNV model based on human case prevalence values. This model explained over 74% of the variation in human prevalence by parish (Table 3). Half of the variables included in the model were thermal indicators (Night_mean, Tmin, and Day_std) or hydrologicical indicators (Precipitation, NDVI_mean, and Diff_min). All of the thermal variables except Tmin had a positive parameter estimates. In other words, an increase of either nighttime mean temperature or daytime temperature standard deviation derived from MODIS data is associated with an increase in human prevalence. An increase in temperature would be expected to decrease the mosquito life span, but would increase virus replication and transmission. Tmin, the minimum temperature derived from the NASA climate grid, had a negative parameter estimate, but was positively correlated (0.34331, p-value = 0.0003) with prevalence. This suggests that a positive increase in Tmin would be expected to result in increased prevalence. The negative parameter estimate is most likely due to interaction with the other variables in defining the slope for prevalence.

Water is a necessary component of the life cycle of mosquito hosts since they are aquatic for most of their non-adult lives. The hydrologic indicators of precipitation, mean NDVI (NDVI_mean), and day-night temperature difference (Diff_min) values derived from MODIS data are indirect indicators of moisture regime. NDVI is related to chlorophyll activity in vegetation and is likely to be higher where more moisture is available to support plant growth. Day-Night temperature difference values are likely to

be lower where moisture is more abundant since water requires more energy than other compounds to undergo a change in temperature. In essence, an increase in water availability would be expected to favor mosquito development and abundance, which in turn favors WNV transmission.

Other environmental factors that were included in the regression model developed were distance from wildlife refuges and distance from streams. Both of these variables were included in at least one other model. For both of these variables, the prevalence increased with an increase in distance from wildlife refuges and streams. These findings suggest that the main WNV development-transmission cycle in birds with human 'spillover' is not occurring in a rural, wildlife-rich setting. Instead, WNV positive dead birds and human cases are more likely to be found in an urban setting, a result echoed by categorical variable chi-square analysis of Louisiana GAP data, and the EPA datalayers forest density (Figure 4) and forest type (Figure 5) which found that WNV positive dead birds and WNV human cases occurred more frequently in urban settings. A multi-year study in Georgia also concluded that urban settings were important for WNV transmission.[97] Urban settings also serve as important breeding ground for *Culex quinquefasciatus*, one of the main vectors for WNV in Louisiana.[102]

Urban in this study is best defined using the description for the 'vegetated urban' category for the LA GAP dataset. It defines 'vegetated urban' as consisting primarily of fringing suburbs and built-up areas of cities, towns, and other urban centers containing sufficient coverages of woody and non-woody vegetation to include urban features (such as buildings, roads and developed areas). Woody or non-woody vegetation is comprised mostly of forest, shrub/scrub or herbaceous plants. Examples include orchards, golf

courses, pasture, gardens, parks, courtyards, decorative shrubbery, abandoned lots containing a variety of vegetation, cemeteries and other possible occurrences of vegetation. In essence, it is defined as vegetation associated with anthropogenic habitats.

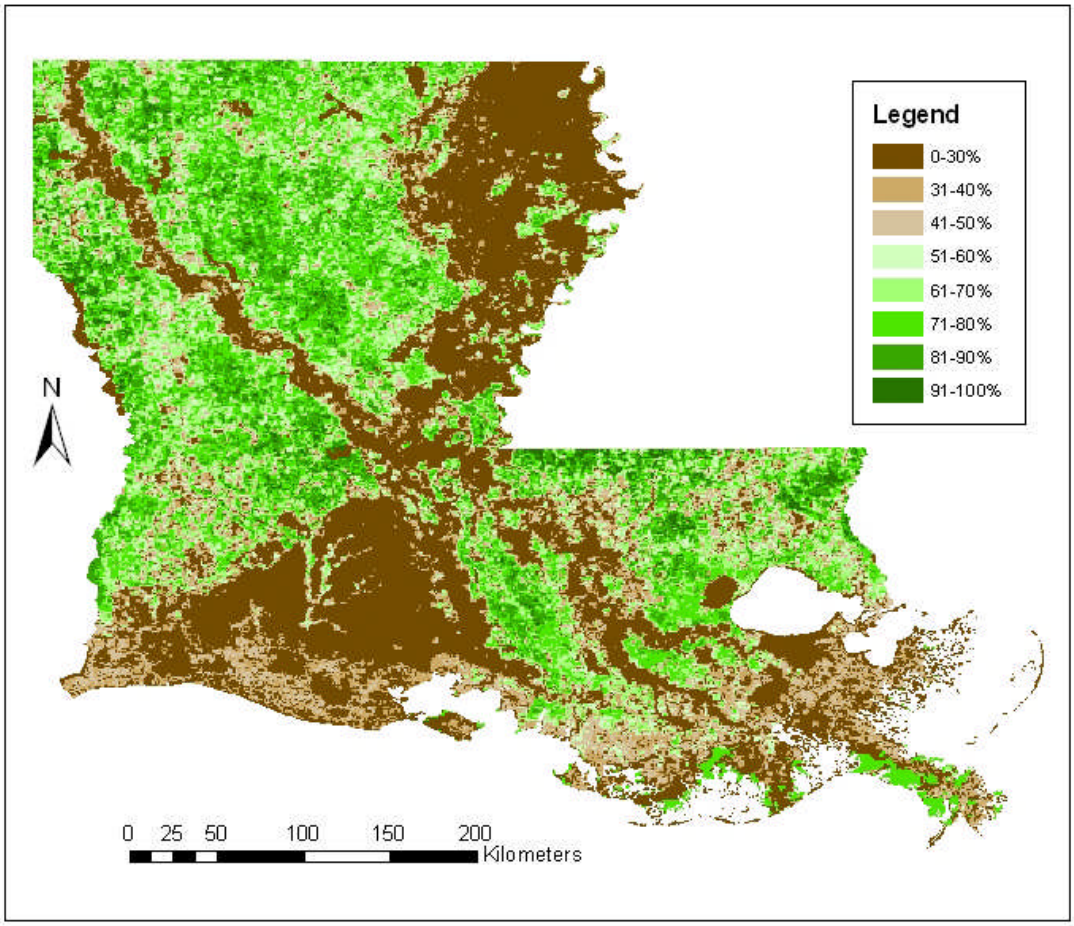

Figure 4: Map showing percent forest for the datalayer Forest Density taken from the EPA website at http://www.epa.gov/airmarkt/cmap/data/category3.html

A separate analysis with only environmental variables produced a regression model from the 2003 human data with an R-square of 0.5386, indicating over 53% of the variation in the human prevalence could be explained by only environmental determinants. Nine variables, including precipitation and distance from wildlife refuges

which were also found in the best bird and human model, were also included in this model. This model emphasizes that WNV transmission is strongly influenced by environmental variables, especially those related to temperature and hydrology.

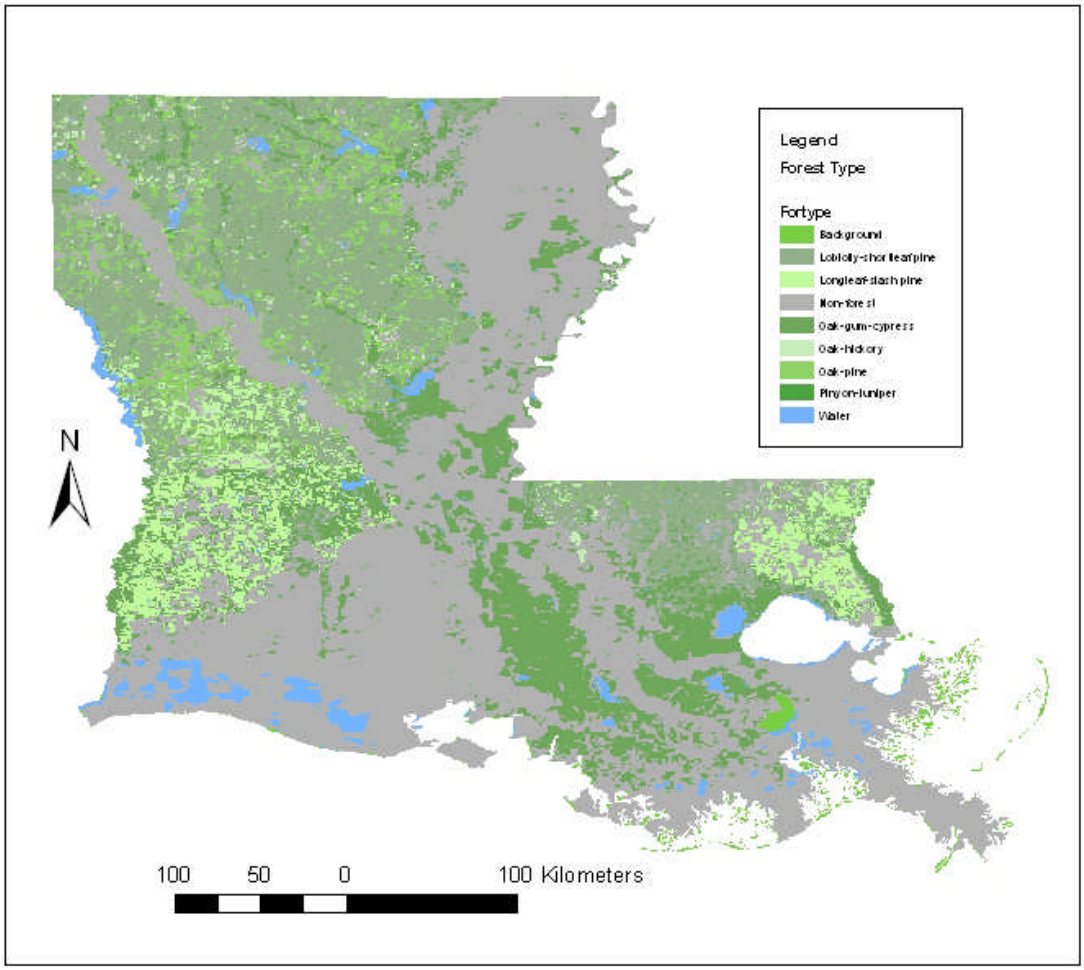

Figure 5: Map showing the categories for the datalayer Forest Type taken from the EPA website at http://www.epa.gov/airmarkt/cmap/data/category3.html

The inclusion of mosquito abatement districts, a non-environmental variable, as a positive parameter estimate into the best bird and human model is surprising since one would think that the presence of mosquito abatement districts would have a negative

impact on prevalence of WNV if they are trying to control the vector population. The reason mosquito abatement districts could be a positive parameter estimate is that the parishes that have mosquito abatement districts usually have a larger urban population, which may come into contact more frequently with WNV spillover from avian amplifiers present in peridomestic settings. This explanation is supported by the finding of more human cases in urban settings, as well as the inclusion of population into the regression model as a positive parameter estimate.

Other analyses showed two geologic categories, 'natural levees' and 'prairie terraces', were found to have higher than expected frequencies when chi-square tests were performed. Natural levees are defined as gray and brown silt, silty clay, some very fine sand, and reddish brown along the Red River. Prairie terraces are defined as light gray to light brown clay, sandy clay, silt, sand, and some gravel. It is unclear how these two geologic categories influence the transmission of WNV, but it is possible that these results were influenced by other factors such as the collapse of the geology categories during analysis or perhaps a function of another variable such as population.

In 2002, WNV was still in the expansion phase and dead birds and human cases were found mainly in the southern part of the state. By contrast, 2003 data revealed the expansion of WNV to the northern part of the state, with human cases concentrated in the Bossier and Shreveport areas. This could by why the x- and y-coordinates are included as significant parameters in the human model since the cases were clustered together. The LS Means procedure demonstrated that the x- and y-coordinates for the human 2003 dataset were significantly different than the coordinates for the both bird datasets and the 2002 human dataset. Cluster analysis would be useful in determining if some of the

differences seen in the 2002 and 2003 models are due to variation in dead bird locations and human cases.

Another possible explanation for the differences seen in 2002 and 2003 bird and human models is that reporting and testing procedures changed between the two years. In 2002, WNV dead bird testing was limited to corvids and birds of prey. In 2003, testing was performed on more species including House Sparrows and Northern Cardinals. Some parishes could have been misclassified as negative if they did not have corvids or birds of prey to test. On the human side, WNV was frequently tested for in patients with mild clinical disease (fever, headaches) in 2002, but 2003 saw over a 10% decrease in the number of patients with mild clinical disease tested. It is possible this decrease could have influenced estimated parish prevalences or affected analysis in other ways since the 2003 human cases had the highest R-square value for all the models. It would be interesting to see if the R-square from the 2002 human model could be improved by removing all West Nile fever cases.

Results reported here for Louisiana have focused on development of a predictive model for risk of disease due to WNV. In order for the best-fitting model to be an operational model for prediction of WNV human disease, it will require validation using similar data from 2004 and subsequent years as independent datasets. Once the model is validated, datalayer extractions would have to be performed on random points or on early cases within a parish at the start of the WNV season so data could be entered into the model to predict the potential prevalence of WNV within that parish. Knowing the potential prevalence of WNV within a parish early on in the transmission season would be useful for several reasons. First, it can guide mosquito abatement districts to be more

or less aggressive in mosquito control and education depending on what the expected prevalence is projected to be. It could also be used to target parishes that may need additional monetary or diagnostic assistance due to increased risk of WNV activity.

In summary, research results reported here have led to the identification of several key factors in the epidemiology of WNV in Louisiana. First, dead birds and human cases were found to be occurring more often in urban settings. This indicates that public health officials and mosquito abatement district workers need to target the urban WNV cycle to control the spillover to humans. This is best accomplished through mosquito abatement efforts that target mosquitoes that transmit WNV.

In addition, warmer temperatures and increases in precipitation have been positively associated with WNV in dead birds and humans. Surveillance and control efforts should be stepped up when there is an increase in temperature and/or an increase in precipitation.

As with risk models developed for other biological systems, these results indicate that the epidemiology of WNV is complex since only 74% of the variation in the human prevalence was explained by the model. Other factors not evaluated in this study, including immunity, awareness, human behavior and variation in bird populations may also play a role in determining the prevalence of WNV in Louisiana. Immuno-competence status could affect which people or birds are more susceptible to disease. Previous reports indicate that IgM antibodies to WNV are present for up to 200 days, suggesting that the WNV cycle may be influenced partly by immunity within the population.[113] Evidence derived from the current study suggests that dead birds and human cases are mostly found in urban environments where WNV awareness is usually

increased due to active mosquito abatement districts and better reporting of WNV surveillance results. People with mild WNV symptoms may not seek medical treatment or diagnosis due to the lack of awareness or surveillance indicators of WNV activity is in their parish. Finally, several bird species have been identified as important amplifiers of WNV in a recent study in Louisiana.[105] One of those birds, northern cardinals, favor urban settings, and hence may not be found in equal proportions across the state, influencing the human prevalence across the state.

The objective of the present study was to explore factors associated with WNV positive dead birds and human cases, but additional surveillance information was collected during the same time frame. Sentinel chicken data, wild bird serosurveys, horse cases, and mosquito pool data were also collected as part of the state surveillance database on WNV in 2001 and subsequent years. This information was not used for two reasons. First, many of the datasets did not include geographic information with sample submissions. If geographic information were collected, this information could be useful in further developing and refining the current models and defining favorable environments and conditions for WNV transmission. Second, not every parish has a mosquito abatement district, limiting the usefulness of this data in a statewide model. Parish surveillance records would be more useful in the development of local models.

The findings in this study reiterate how important environmental variables, especially thermal and hydrologic indicators, are in affecting WNV transmission. Increases in either of these can increase WNV prevalence. In addition, the findings in this study indicated that WNV spillover from avian hosts to accidental human hosts occurs predominantly in urban settings. To break the transmission of WNV to humans,

mosquito abatement districts and public health officials must focus control measures and education efforts in reducing mosquito populations in these urbanized settings.

Future studies utilizing both WNV positive and negative data from additional sources such as mosquito and sentinel bird data are needed on the parish (county) level or using higher resolution data at the city level to identify specific features or microhabitats that make urban settings ideal for WNV activity. In addition, studies are needed to answer the question if WNV is truly absent in some parishes or is it a function of underreporting dead birds and human cases due to a lack of awareness.

SEROPREVALENCE OF WEST NILE VIRUS IN WILD-CAUGHT BIRDS IN EAST BATON ROUGE PARISH, LOUISIANA

Introduction

West Nile virus (WNV), a member of the family Flaviviridae and genus Flavivirus, was first isolated from a febrile woman in Uganda in 1937.[16] Since that initial isolation, WNV has been found in many parts of the world including Africa, Europe, Asia, and the Middle East.[20,22-24] Since the early 1950s, wild birds have been recognized as playing an important part in the epidemiology of WNV. Wild birds are the main reservoirs for WNV and are also thought to be responsible for disseminating WNV to new areas.[16] Since birds are the primary reservoirs for WNV, they are a useful tool for monitoring WNV activity. In Karoo, South Africa, an increase in the prevalence of WNV was recorded at the same time as a large human outbreak in 1974.[19,21] Countries such as Romania, Slovakia, Pakistan and others have employed wild birds as well as sentinel chickens for WNV surveillance.[22,24,26]

In 1999, WNV emerged in New York City. Within a matter of five years, it disseminated across the entire continental United States. In 2001, WNV activity was first reported in Louisiana. One human case, ten equine cases, and six WNV positive birds were recorded.[8] In "The Arboviruses: Epidemiology and Ecology," Theodore F. Tsai and Carl J. Mitchell describe the SLE cycle for eastern states as involving birds that are often abundant in urban-suburban environments, which has also been the case with several WNV serosurveys in birds.[2] They suggest that the peridomestic habits of these birds, along with similar habits of main host mosquitoes, could result in a SLE virus cycle in backyards with spillover into humans and other species. If this is the case with SLE, it could also possibly be true with WNV.

In 2002, Louisiana had 329 confirmed WNV human cases, which translated to a case rate of 7.362/100,000 people and a mortality rate of 0.559/100,000 and was among the highest in the United States. In order to understand the epidemiology of WNV in Louisiana, especially in East Baton Rouge (EBR) Parish, a study using wild-caught birds was initiated. It was hypothesized that certain bird species or sites would have a higher prevalence of WNV than the overall prevalence for the area, and would provide information on bird species and locations that were important in WNV transmission. To test this hypothesis, wild birds were caught with mist nets at sites in EBR Parish, Louisiana. These sites, many of which were located around parks or in residential neighborhoods, were chosen in cooperation with East Baton Rouge Mosquito and Rodent Control (EBRMARC), the entity which conducts arboviral surveillance within the parish. Bird sera samples were tested with the plaque reduction neutralization test (PRNT),the "gold standard" for antibody testing, to determine which birds were exposed to WNV.

Methods and Materials

Blood was collected from wild birds from November 2002 until October 2004 by EBRMARC as part of their routine surveillance for arboviruses. Birds were collected from a total of 41 sites in Baton Rouge (30.4581° N, -91.1402° W) and surrounding EBR Parish during the two-year study with up to 15 of those sites trapped per month (see Appendix C for map). A total of 1297 blood samples were collected, but only 1287 samples were tested due to insufficient quantity of serum in 10 samples. Samples were collected from 48 different species of birds. Gender, age, species, date bled, band number, and collection site were recorded for each bird. Age was defined as juvenile (born in year captured) or adults (born prior to year captured). In some instances age or

gender could not be determined and were recorded as unknown. Band numbers indicated seven birds were recaptured during the study.

No more than 0.5ml of blood was collected from each of the birds from the jugular vein. Blood was placed into clot activator and gel tubes (BD Microtainer™, Franklin Lakes, NJ). Samples were refrigerated at 4°C until they transported on ice to Louisiana State University School of Veterinary Medicine. Once samples arrived, they were centrifuged at 2000rpm for 10 minutes. Serum was removed from each sample and pipetted into a 1.5ml microcentrifuge tube. After being pipetted into the microcentrifuge tube, the serum samples were heat inactivated in a 56°C water bath for 30 minutes to remove nonspecific inhibitors. Samples were tested for neutralizing antibodies to WNV and SLE virus by the PRNT.

Virus Stock

WNV antigen was prepared by diluting virus with BA-1 diluent (see Appendix A) to 200 plaque forming units (PFU) /100µl. Virus titer was established in the following manner. First, WNV isolated from a blue jay in 2001 was added to a monolayer of Vero cells in a flask at a 1:5 concentration in BA-1. The virus and the Vero cells were incubated together at 37°C, 5% CO2 for 90 minutes. After incubation, the viral inoculum was removed. Media containing minimum essential media (MEM) plus 200IU/ml penicillin, 100mg/ml streptomycin, 10µg/ml amphotericin B, and 5% fetal bovine serum was added to the cells. The Vero cell monolayer was monitored daily for cytopathic effects (CPE). CPE was usually observed within 48 hours. After CPE was observed, media and cells were removed from the flask and then centrifuged for 10 minutes at 2000rpm. Virus was then divided into 100µl aliquots.

One of the viral aliquots was diluted with a phosphate-buffered saline (PBS) from 10^{-1} to 10^{-9}. One hundred microliters of virus dilutions from 10^{-3} to 10^{-9} were added in triplicate to a 24-well plate covered with Vero cells. One hundred microliters of PBS was also added to three wells to serve as a control. The 24-well plate with the viral dilutions and PBS controls was covered and allowed to incubate for 90 minutes at 37°C, 5% CO_2. After incubation, the wells were drained of media and virus mixtures. A 2% carboxymethylcellulose overlay (see Appendix A) was then added to each well. The plate was covered, wrapped in parafilm, and allowed to incubate for 48 hours.

After 48 hours, the plate was removed from the incubator, and the parafilm was removed and 900µl of formalin was added to each well to fix the Vero cells. Formalin was left on the cells for a minimum of 30 minutes. The formalin and media were removed from the wells and the plates were then washed with warm water to remove remaining media. After removing the media, roughly 200µl of crystal violet Gram stain was added to each well to aid in the visualization of plaques. Crystal violet Gram stain was left in the wells for at least one minute. Plates were then rinsed with warm water. After rinsing the plates, plaques were visualized using a radiology light box. A virus titer was calculated by counting plaques at the virus dilution that yielded between 20 and 200 plaques per well. The three wells at that dilution were counted and averaged to establish a working stock titer. The antilog of the WNV working stock was then calculated to form a dilution scheme to reach 200PFU/100µl.

Plaque Reduction Neutralization Test

For the initial phase of the plaque reduction neutralization test, 20µl of heat inactivated serum was diluted in 80µl of BA-1 diluent in an ELISA plate well. Next,

50µl of serum and BA-1 diluent from the first well was added to 50µl of BA-1 diluent in the next well. This was repeated four additional times to make serum dilutions of 1:5, 1:10, 1:20, 1:40, 1:80, and 1:160. A volume of 50µl of diluted virus was added to each serum dilution to produce final serum dilutions of 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320 with 100 virus plaques added to each dilution. At this time, virus was also diluted to 100 PFU/100µl, 10 PFU/100µl, and 1 PFU/100µl to act as backtitration to establish a 90% virus neutralization plaque count. Serum virus mixtures were incubated for 60 minutes at 37°C, 5% CO_2.

After 60 minutes, serum virus mixtures and the backtitration were removed from the incubator. Six-well plates with Vero cells were drained of media, leaving only 300-400µl of media in each well. After wells were drained, serum virus mixtures were added to the wells with each sample getting its own six-well plate. The backtitration plate was made by adding 100µl of BA-1 diluent to one well, 100µl of 1 PFU/100µl to one well, 100µl of 10 PFU/100µl to two different wells, 100µl of 100 PFU/100µl to one well, and 50µl of 200 PFU/100µl to one well. Plates were then put into the incubator for 90 minutes. Plates were swirled every 30 minutes to prevent the Vero cell monolayer in the six well plates from drying out.

After 90 minutes, plates were removed from the incubator and the media drained. Three milliliters of an overlay containing 2X-M199 (see Appendix A) and 2% UltraPure™ Agarose (Invitrogen, Carlsbad, CA) were then added. Plates were then placed in the incubator for 48 hours. After the 48 hour incubation, a second overlay was added. Two milliliters of overlay with 2X-M199, 2% agarose, and neutral red solution (Sigma-Aldrich, St. Louis, MO) was added to each well to aid in visualization of the

plaques. Plates were then incubated for another 24 hours. After incubation, plates were removed and the plaques counted. Plaques counted for each sample were compared to the average of the two 10 PFU/100µl wells from the backtitration since this value represented neutralization of 90% of the viral plaques added to each serum dilution. A sample was considered to have neutralizing WNV antibodies if the count in the sample's wells were less than or equal to the average of the two 10 PFU/100µl wells. Titers were expressed as the reciprocal of the serum dilution reducing the number of plaques by ≥ 90%. A sample was considered negative if the first dilution did not have any neutralizing antibodies.

SLE working stock was grown in a manner similar to WNV with one exception. The SLE working stock was incubated for seven days instead of 48 hours since SLE grows slower than WNV. The six-well plates were incubated for six days before adding the second overlay with neutral red. Many of the serum samples were screened for SLE neutralizing antibodies starting at a 1:20 dilution since insufficient quantities of serum remained to test at a 1:10 dilution after being used to test for WNV neutralizing antibodies. Samples that had WNV titers ≥ 1:10 without SLE titers were considered WNV positive.

Statistical Analysis

Seroprevalences were calculated for species, location, age, gender, and month. Ninety-five percent confidence intervals (95% CI) were constructed for all groups that had a (prevalence) x (# of birds tested) greater than five to see if there was any overlap with the overall prevalence. A one sample chi-square test was performed for non-overlapping 95% CI to see if differences were significant from the overall

seroprevalence. The expected value was calculated by taking the number of birds caught and multiplying it by the overall prevalence. Test statistics were compared to one sample chi-square value of 3.84, which corresponds to a p-value of 0.05.

Proc GLM with Least Squares (LS) Means was performed on the natural log transformations of the antibody titers by gender, age, and month to make comparisons between the groups. LS Means was also used to compare geometric mean antibody titers for species and locations that had a sample size of at least five negative and five positive WNV birds. Serostatus was used as an interaction effect to compare the geometric mean titers of antibody positive and negative birds. Tukey post hoc tests were performed on groups to examine significant differences between the groups.

Results

Overall, 222/1287 (17.25%, CI: 15.19, 19.31) samples tested positive for WNV antibodies. The seroprevalence and 95% CIs for WNV antibodies in birds captured in this study are shown by species (Table 6) and location (Table 7). Seven birds were recaptured during the study. Of the seven birds recaptured, five of the birds did not test positive for WNV during the course of the study, one bird had an antibody titer of 1:80 in January 2003, and then had a titer of ≥1:320 when recaptured in February 2004, while another bird had a 1:80 titer on captures four months apart. In addition, five birds were SLE positive while one bird had an ambivalent Flavivirus result.

The yellow-bellied sapsucker (*Sphyrapicus varius*) had the highest prevalence at 100%, but this result is misleading since the only one yellow-bellied sapsucker was captured. Mourning doves (*Zenaida macroura*), northern cardinals (*Cardinalis cardinalis*), Northern Mockingbird (*Mimus polyglottos*), and red-wing blackbirds

Table 6: WNV Seroprevalence and 95% Confidence Interval by Species in Birds at 41 sites in East Baton Rouge Parish from November 2002 to October 2004

Species	# Positive	# Tested	Prevalence	95% CI
American Goldfinch[W]	0	6	0.00%	
American Redstart[S]	0	2	0.00%	
American Robin[R]	0	4	0.00%	
Blue Grosbeak[S]	0	1	0.00%	
Blue Jay[R]	3	21	14.29%	
Brown Thrasher[R]	4	21	19.05%	
Brown-headed Cowbird[R]	3	40	7.50%	
Carolina Chickadee[R]	1	29	3.45%	
Carolina Wren[R]	5	30	16.67%	(3.29, 30.05)
Chipping Sparrow[W]	0	8	0.00%	
Common Grackle[R]	3	18	16.67%	
Common Yellowthroat[R]	0	2	0.00%	
Eastern Phoebe[W]	0	1	0.00%	
European Starling[R]	0	2	0.00%	
Field Sparrow[R]	0	4	0.00%	
Gray Catbird[R]	0	1	0.00%	
Hairy Woodpecker[R]	0	1	0.00%	
Hermit Thrush[W]	1	5	20.00%	
Hooded Warbler[S]	0	4	0.00%	
House Finch[R]	0	2	0.00%	
House Sparrow[R]	61	311	19.61%	(15.2, 24.02)
Indigo Bunting[S]	0	3	0.00%	
Louisiana Waterthrush[S]	0	1	0.00%	
Mourning Dove[R]	8	23	34.78%	(15.32, 54.24)
Northern Cardinal[R]	104	404	25.74%	(21.48, 30.00)*
Northern Junco[W]	0	1	0.00%	
Northern Mockingbird[R]	8	20	40.00%	(18.53, 61.47)
Orchard Oriole[S]	0	3	0.00%	
Pine Warbler[R]	0	1	0.00%	
Prothonotary Warbler[S]	0	2	0.00%	
Purple Martin[S]	1	18	5.56%	
Red-bellied Woodpecker[R]	0	7	0.00%	
Red-headed Woodpecker[R]	0	2	0.00%	
Red-wing Blackbird[R]	7	28	25.00%	(8.97, 41.03)
Ruby-crowned Kinglet[W]	0	8	0.00%	
Rufous-sided Towhee[W]	1	11	9.09%	
Savannah Sparrow[W]	1	19	5.26%	
Swainson's Warbler[S]	0	2	0.00%	
Tufted Titmouse[R]	1	36	2.78%	
Unknown	0	3	0.00%	
Unknown Yellow-rumped Warbler	0	4	0.00%	
White-throated Sparrow[W]	8	162	4.94%	(1.60, 8.28)*
White-eyed Vireo[R]	0	2	0.00%	
Wilson's Warbler[W]	0	1	0.00%	
Wood Thrush[S]	0	1	0.00%	
Yellow-rumped Warbler[W]	1	10	10.00%	
Yellow-bellied Sapsucker[W]	1	1	100.00%	
Yellow-breasted Chat[S]	0	1	0.00%	
Overall (total)	222	1287	17.25%	(15.19, 19.31)

R, resident year-round; S, summer resident; W, winter resident; *Significantly different from overall prevalence; See Appendix B for scientific names.

(*Agelaius phoeniceus*) also had high prevalences but with large sample sizes. Northern cardinals (25.74%; CI: 21.48, 30.00%) had a higher seroprevalence than the overall prevalence (17.25%; CI: 15.19, 19.31%) while white-throated sparrows (*Zonotrichia albicollis*) (4.94%; CI: 1.60, 8.28%) had a lower seroprevalence than the overall seroprevelance. One sample chi-square tests for each of these species confirmed these seroprevalences were statistically different ($p < 0.05$) from the overall seroprevalence.

The LS Means procedure was performed on Carolina wrens, house sparrows, northern cardinals, red-wing blackbirds, and white-throated sparrows. Statistical analysis revealed that WNV positive house sparrows had the highest geometric titer of 5.1 (antibody titer of 1:164), but was not significantly different ($p \geq 0.674$) from WNV positive Carolina wrens or WNV positive northern cardinals. WNV positive white-throated sparrows had the lowest geometric mean titer (2.39; antibody titer of 1:11) and were significantly different ($p \leq 0.0023$) from the other species regardless of serostatus.

Several locations, including City Park, Drusilla Park, Highland Rd, Hooper Rd Park, and Pecue Lane, had a high percentage of birds testing positive for WNV antibodies. Confidence intervals were constructed for all sites that had a (prevalence) x (# of birds tested) greater than five to look for significant differences between sites. Of the sites that had a 95% CI calculated, only City Park (35.71%; CI: 21.22, 50.20), Drusilla Park (50.00%; CI: 26.9, 73.1), Highland Rd (42.86%; CI: 26.46, 59.26), and Pecue Lane (35.59%, CI: 23.18, 48.00) had CI that did not overlap with the overall prevalence. A one sample chi-square test performed for City Park, Drusilla Park, Highland Rd, and Pecue Lane demonstrated that all of these locations had seroprevalences that were statistically different ($p < 0.05$) from the overall seroprevalence.

Table 7: WNV Seroprevalence and 95% Confidence Interval by Location in 49 Species Captured in East Baton Rouge Parish from November 2002 to October 2004

Location	# Positive	# Tested	Prevalence	95% CI
Bickham Rd	5	52	9.62%	(1.61, 17.63)
Blackwater Rd	11	42	26.19%	(12.89, 39.49)
BREC Horse Center	9	65	13.85%	(5.45, 22.25)
Carpenter Rd	11	37	29.73%	(15, 44.46)
Cedar Ridge Park	3	14	21.43%	
Centurion Place	1	8	12.50%	
City Park	15	42	35.71%	(21.22, 50.2)*
Clark St	8	66	12.12%	(4.25, 19.99)
Comite Dr	0	14	0.00%	
Denham Rd	1	30	3.33%	
Doyle Bayou Park	2	12	16.67%	
Drusilla Park	9	18	50.00%	(26.9, 73.1)*
EBRMARC	3	22	13.64%	
Ednie Dr	1	35	2.86%	
Emmett Bourgeios Rd	7	43	16.28%	(5.25, 27.31)
Evangeline Fire Station	2	8	25.00%	
Greenwell Springs Park	10	36	27.78%	(13.15, 42.41)
Greenwell Springs Rd	9	31	29.03%	(13.05, 44.83)
Greenwood Park	3	27	11.11%	
Highland Rd	15	35	42.86%	(26.46, 59.26)*
Hoo Shoo Too Rd	4	23	17.39%	
Hooper Rd Park	3	5	60.00%	
Hwy 64 at Blackwater Rd	2	11	18.18%	
Lazy B Stables	5	44	11.36%	(1.98, 20.74)
Lee High School	2	42	4.76%	
Liberty Rd	4	35	11.43%	
LSU Swine Unit	4	31	12.90%	
MacHost Rd	9	71	12.68%	(4.94, 20.42)
O'Neal Lane	2	17	11.76%	
Parklawn Park	2	10	20.00%	
Pecue Lane	21	59	35.59%	(23.18, 48.00)*
Riley Rd	5	47	10.64%	(1.82, 19.46)
S. Magnolia Park	3	24	12.50%	
Southern University	12	52	23.08%	(11.63, 34.53)
Strain Rd	4	20	20.00%	
Sunshine Rd	1	29	3.45%	
Tristian Ave Park	2	12	16.67%	
Welcome Heights	0	2	0.00%	
White Bayou	3	40	7.50%	
Willow Dr	6	48	12.50%	(3.14, 21.86)
Zoo Entrance	3	28	10.71%	
Overall (total)	222	1287	17.25%	(15.19, 19.31)

*Significantly difference from overall prevalence; See Appendix C for map.

The LS Means procedure analysis was performed on 11 locations. Positive birds at Lazy B Stables had the highest geometric mean titer of 5.91 (antibody titer of 1:369) and were statistically different ($p \leq 0.0153$) from all the other sites. Other sites had positive mean geometric titers ranging from 3.46-5.2 (antibody titers of 1:32-1:181).

Seroprevalences for age, gender, and month were also calculated. For age, unknowns (29.4%, CI: 18.6, 40.2%) had a higher seroprevalence than either adults (18.2%, CI: 15.8, 20.8%) or juveniles (11%, CI: 7.3, 14.6%). For gender, males (23.5%, CI: 18.9, 28.1%) had a higher seroprevalence than either females (19%, CI: 15.8, 22.3%) or unknowns (9.9%, CI: 7, 12.8%). The months January (24.6%, CI: 13.8, 35.4%), April (25.6%, CI: 17.8, 33.4%), June (18%, CI: 11.6, 24.4%), August (25%, CI 15.5, 34.5%), and October (29.3%, CI: 19, 39.6%) had a higher seroprevalence than the overall prevalence, but all of these months did overlap with the 95% CI for the overall prevalence.

The LS Means procedure revealed that juvenile positive birds had the highest mean titer at 5.46 (antibody titer of 1:234) and the Tukey post hoc pairwise comparisons showed this group to be significantly different ($p \leq 0.007$) from all the other groups (Figure 6). The other positive age groups, adults and unknowns, were significantly different from each other ($p = 0.0015$).

The LS Means procedure demonstrated that positive females had the highest mean titer at 5.07 (antibody titer of 1:159) and were significantly different ($p \leq 0.0006$) from all groups (Figure 7). The other groups, males and unknowns, were significantly different ($p \leq 0.0006$) from each other.

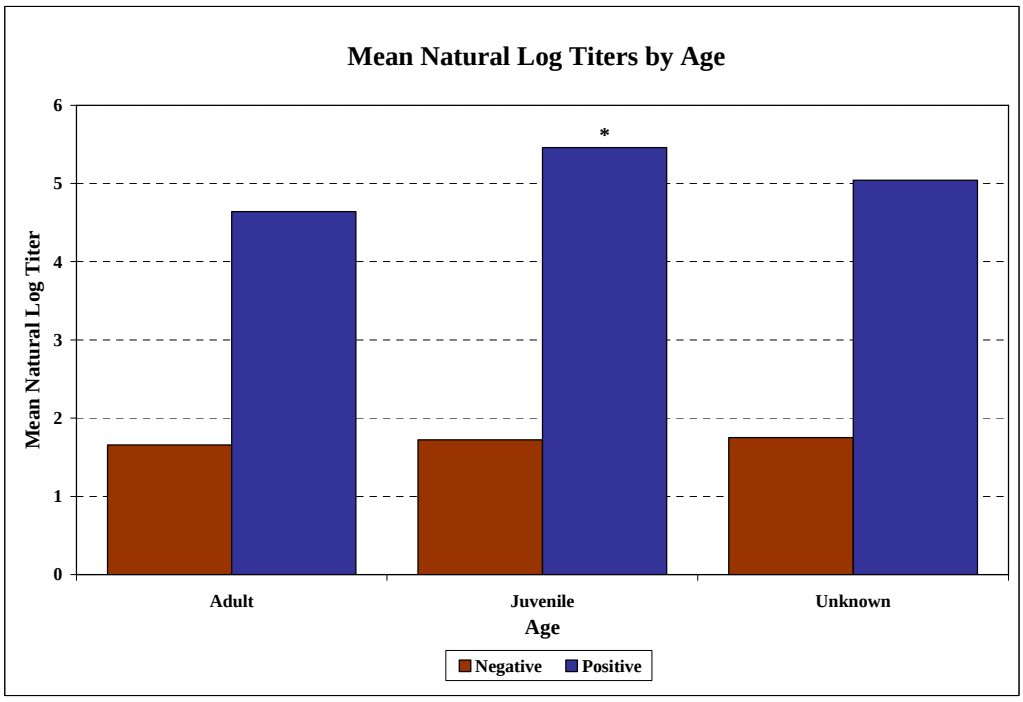

Figure 6: Geometric mean natural log antibody titers for WNV by age and serostatus for birds captured in East Baton Rouge Parish from November 2002 to October 2004. *Significantly different from other age titers

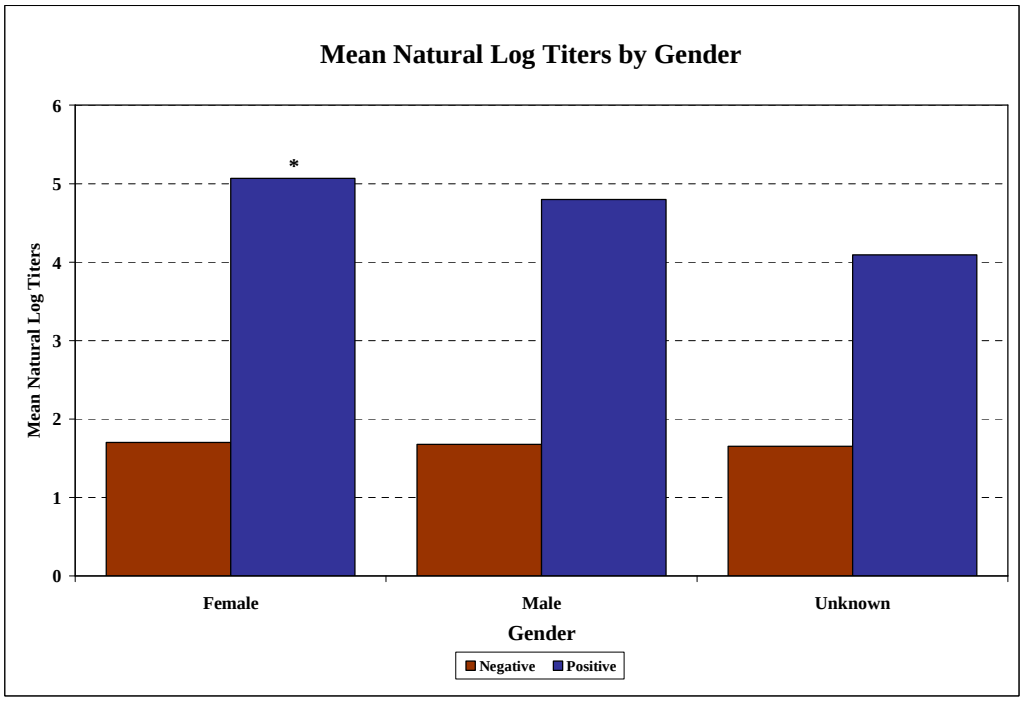

Figure 7: Geometric mean natural log antibody titers for WNV by gender and serostatus for birds captured in East Baton Rouge Parish from November 2002 to October 2002. *Significantly different from other gender titers

The LS Means analysis for months demonstrated that WNV positive birds had the highest geometric titer of 5.72 (antibody titer of 1:305) in September which was statistically different ($p \leq 0.0016$) from every other month except July which had a geometric mean titer of 5.39 (antibody titer of 1:219). From July to October, WNV positive wild birds averaged a geometric mean titer greater than five (antibody titer of 1:148). WNV positive birds caught in January and December has significantly lower geometric mean titers at 3.92 (antibody titer of 1:50) and 4.04 (antibody titer of 1:59), respectively (Figure 8).

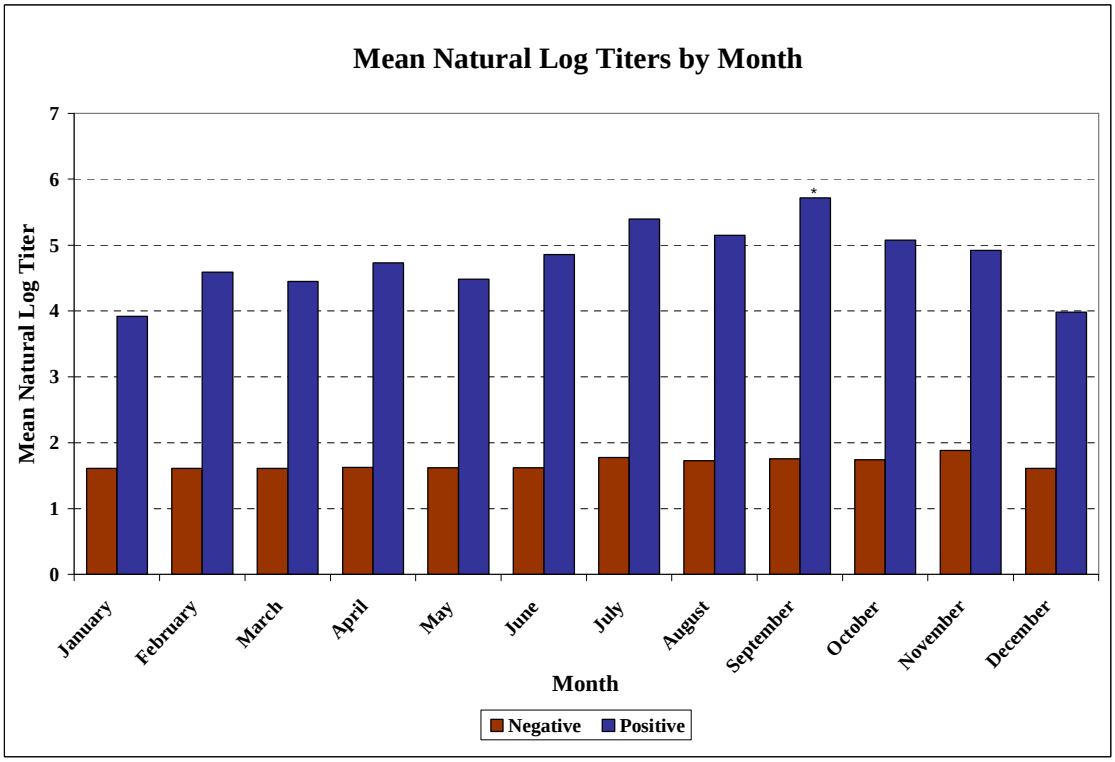

Figure 8: Geometric mean natural log antibody titers for WNV by month and serostatus for birds captured in East Baton Rouge Parish from November 2002 to October 2004.
*Significantly different from all other month titers except Positive July

Discussion

In terms of other serosurveys in the United States, the seroprevalence of 17.25% reported here is higher compared to reports of wild caught birds by Florida in 2001 (10.5%), Illinois in 2002 (5.3%), and California in 2003 (1.6%).[94,96,114] A multi-year serosurvey conducted in Georgia from 2000-2004 had a seroprevalence of 6.2% (869/14,077) while another multi-year study in Illinois from 2001-2004 had a seroprevalence of 6.6% (348/5,236).[97,98]

The higher seroprevalence observed in this study could be caused by several factors. First, testing strategies were different. Louisiana used PRNT testing, while the two Illinois studies used ELISA, and the California survey used an enzyme immunoassay (EIA) to detect WNV antibodies. Differences in test sensitivity could result in misclassification and under/over estimation of seroprevalence. A study comparing ELISA with PRNT showed a kappa statistic of 0.91, suggesting that the tests results generally agree, but that some of the birds could be misclassified as false negatives or false positives using the ELISA test.[115] The EIA used for screening birds in California was modified from previous EIA tests developed for Western equine encephalitis and SLE. The sensitivity for the SLE EIA was 71%.[88]

A second explanation for the higher prevalence here was that Illinois and California conducted some of their surveillance in highly urbanized areas, including downtown Chicago and Los Angeles, respectively. The California study remarked that surveillance methods did not work well in urban or periurban areas. Many of the locations in EBR Parish were in parks or highly wooded areas.

A third potential explanation for the higher seroprevalence in this study is that the climate in Louisiana greatly differs from the climate in Illinois and California. For example, the climate in Louisiana supports year round arboviral transmission unlike northern states. A fourth and final explanation for the higher prevalence in Louisiana was that this study looked at WNV antibody titers over several years. Antibodies could persist for an indefinite length of time in birds, thus increasing the prevalence of WNV over time. A study in Georgia recently found that WNV antibodies persisted for sixty weeks.[115] It must also be remembered that antibodies only indicate exposure and cannot indicate a specific location or time that a bird became infected with WNV.

The overall seroprevalence of 17.25% observed in the present study was lower than a study conducted in St. Tammany Parish, Louisiana in 2002 (24.7%, CI 20.1-29.9%) that tested birds with PRNT.[105] This could have been because the St. Tammany study only sampled birds in August and October, so only seven (1.6%) nonbreeder birds (birds that do not nest in Louisiana during spring) were tested during the St. Tammany study compared to 230 (17.9%) in this study. Of the seven nonbreeder birds tested in the St. Tammany study, none were positive for WNV.

The northern cardinal (*Cardinalis cardinalis*) and white-throated sparrow (*Zonotrichia albicollis*) had seroprevalences that were significantly different from the overall prevalence of wild birds caught and tested in this study. Northern cardinals had a higher seroprevalence that has been seen in other studies, including the previously mentioned California, Florida and Georgia serosurveys or the serosurvey in St. Tammany Parish, Louisiana.[94,96,98,105] Northern cardinals are nonmigratory species that live in residential and rural deciduous wooded areas. They are continuously exposed to

arbovirus activity in Louisiana. In a recent paper, northern cardinals were identified as important amplifying hosts due to high levels of exposure, moderate to high abundances, and experimentally demonstrated high host competence.[105] In addition to being important amplifying hosts, the peridomestic nature and high seroprevalence of northern cardinals to WNV mimic the spillover conditions necessary for SLE human infections and could be one of the reasons that humans develop WNV infections.[2]

White-throated Sparrows, on the other hand, are a migratory species that live in the south during the winter and return to the north in spring, hence they are only exposed to arbovirus activity in Louisiana from October to April. This finding also coincides with the finding in the multi-year Illinois study that found birds breeding in Illinois had a higher seroprevelance than transient species.[97]

The high geometric mean titers of positive Carolina wrens, house sparrows, and northern cardinals could indicate antibody persistence in these species, which could confound interpretation of the seroprevalences reported for these species. The low geometric mean titer of positive white-throated sparrows could result in the lower prevalence seen in these birds if antibodies are of short duration. These two features of white-throated sparrows might make them an ideal wild bird sentinel for new hot spots of WNV infection. More studies are warranted to study antibody persistence in these species.

One bird, a northern cardinal, had antibody titers of 1:80 on two different captures four months apart, while another bird, a brown-headed cowbird, had an increase in titer from 1:80 in January 2003 to a titer of ≥1:320 when recaptured in February 2004. The northern cardinal reinforces previous research which has shown the persistence of

antibodies in birds.[115] The increase in the titer of the brown-headed cowbird raises an interesting question. WNV antibodies have been shown to persist in birds for over a year, but it has not been shown if antibodies provide life-long immunity against future infections.[115] This increased titer could have been associated with: 1) a second exposure re-stimulating antibody production, or 2) a new exposure and new humoral response, a recurrent latent infection with the original virus.

The four sites, City Park, Drusilla Park, Highland Rd, and Pecue Lane that have a higher seroprevalence than the overall seroprevalence are located near small wooded lots or deciduous trees. City Park, Drusilla Park, and Pecue Lane are also located near sources of water, including drainage ditches and canals. The locations are prime mosquito habitats, especially for *Culex quinquefasciatus* which thrive in polluted habitats, and may be associated with the higher seroprevalences at these sites. A comparison to mosquito trap findings would be needed to explore this hypothesis.

Results of the age, gender, and month analyses results are interesting from several standpoints. First of all, adults had a higher seroprevalence than juvenile birds, but juvenile or hatching year birds had a higher geometric mean titer. The higher seroprevalence in adult birds is in agreement with the multi-year Illinois study. This phenomenon could be due to the persistence of antibodies in adult birds, which have a greater potential to be exposed over time (longevity).[97,107] The higher geometric mean titers in the juveniles is likely associated with the fact that juveniles were exposed to WNV the year they were caught, while adults could have been exposed at any point in their lives since the introduction of WNV into Louisiana and may experience a gradual waning of antibodies.

The influence of gender of birds has rarely been addressed in previous serosurveys for WNV as a factor influencing potential exposure. Males had a higher seroprevalence than females, but females had a higher a higher geometric mean titer than males. The higher seroprevalence in males could be due to the larger home ranges which may increase their chances for exposure. The higher titers in positive females could be due to nesting behaviors which may cause them to be repeatedly exposed to WNV infected mosquitoes or they could possibly be exposed through WNV-infected nestling birds.

The high serprevalences in January, April, June, August, and October may be a function of species and locations for these months, since there does not appear to be a pattern. Not every site was trapped every month, and sample collection was influenced in part by human and dead bird activity since EBRMARC relied on this information for mosquito control purposes. The higher geometric mean titers reported in positive birds trapped from July-October coincide with other indicators of WNV activity. Greater WNV activity among humans, dead birds, and mosquitoes is usually seen in the summer. The lower mean titers in January and December could indicate less WNV activity, the waning of antibodies from summer infections, or the inclusion of non-resident birds in the study. The low geometric mean titer of positive WNV birds coupled with the high seroprevalence of birds seen in January suggest that antibodies do persist for an undefined period of time, but more studies are needed to see if the higher geometric mean titers indicate a recent infection or if these high titers can persist for an extended amount of time.

The findings in this study emphasize that seroprevalence is relative to a number of factors including age, resident status, species, and time of year. Any of these factors can greatly affect seroprevalence and emphasize the need for WNV wild bird surveillance to concentrate on testing juvenile birds and/or testing for WNV antigen for information on current WNV activity to institute WNV control measures.

ORAL INOCULATION OF MEDITERRANEAN HOUSE GECKOS (*HEMIDACTYLUS TURCICUS*) WITH WEST NILE VIRUS

Introduction

Previous research has shown ectothermic vertebrates may be suitable hosts for arbovirus infections. Field studies have revealed antibodies to Japanese encephalitis (JE) virus in various species of snakes, lizards, and turtles in Asia.[40,116,117] Laboratory studies have shown that JE virus can replicate in lizards and snakes in Asia.[40,118] Other laboratory experiments with garter snakes (*Thamnophis* spp.) and Texas tortoises (*Gopherus berlanieri*) in the United States indicated they were competent reservoirs for Western equine encephalitis virus.[116,119] Finally, St. Louis encephalitis (SLE) virus neutralizing antibodies were detected in a painted turtle (*Chrysemys picta picta*) and a leopard frog (*Rana pipiens pipiens*) in New York.[116]

WNV was first detected in Uganda in 1937, and for most of the 20th century was limited to Europe, Asia, and Africa. In 1999, WNV emerged in North America as an important pathogen and has spread rapidly across the country, presenting a threat to both human and animal health. WNV was first found in Louisiana in 2001. A WNV epidemic followed in 2002 with 329 human cases and 25 deaths.[8]

Very few studies have been performed on ectotherms to determine their role in the epidemiology of WNV. One of the earliest studies involving ectotherms and WNV revealed that the lake frog (*Rana ridibunda*) in Russia produces a WNV viremia capable of infecting mosquitoes.[19,24] Antibodies have been found in Nile Crocodiles (*Crocodylus niloticus*).[19,24] An experimental inoculation trial in the United States with bullfrogs (*Rana catesbeiana*), Florida garter snakes (*Thamnophis sirtalis sirtalis*), and green iguanas (*Iguana iguana*) showed that they became infected, but only the bullfrogs and the

iguanas developed a viremia.[116] Starting in 2001, WNV was reported as causing severe economic losses for alligator farms. Further studies showed that alligators produce a high enough viremia to infect mosquitoes, other alligators, and humans.[40,41] In fact, one human case of WN fever has been linked to a WNV outbreak in juvenile American alligators (*Alligator mississippiensis*).

Louisiana is home to a variety of reptiles, including turtles, alligators, snakes, and lizards because of its semitropical climate. The Mediterranean house gecko (MHG) (*Hemidactylus turcicus*) was first introduced into the United States through Florida during the 20th century.[119] The gecko is now considered naturalized to Louisiana. These reptiles are routinely observed on human dwellings and other buildings during the summer months, while cooler winter temperatures force the geckos indoors. Because the gecko is an insectivore, it is possible that it could become exposed to WNV if it consumed a positive vector, especially since it is commonly found in WNV-positive areas. The purpose of this study was to determine if MHG can be infected orally with WNV.

Methods and Materials

Forty-three adult MHG were purchased from a reptile collector (Mr. Danny Elder, LaPlace, Louisiana, 30.07503 °N, -90.4849 °W) and transported to the LSU vivarium. The exact location these animals were collected is unknown, but they were collected in John the Baptist Parish, Louisiana. The geckos were housed individually with escape proof lids and maintained at an environmental temperature between 82-84°F (27.8-28.9°C), a temperature similar to an experimental WNV infection trial with alligators. The geckos were left in the cages for seven days to acclimate. The geckos were fed

mealworms and misted twice daily with chlorinated water. After the acclimation period, the geckos were randomly assorted into two groups: Group 1 (control: n=23) and group 2 (inoculated: n=20). The two different groups were housed in separate rooms each with their own ventilation. Gecko number 9 died shortly after its arrival. Because of the small size of the geckos, blood could not be collected from them prior to initiating the research to determine their WNV status. Instead, the control group was intended to demonstrate negativity. The geckos in the inoculated group were given 10^6 plaque forming units (PFU) WNV (0.1 ml) orally to simulate the ingestion of a positive mosquito.[41,120-2] The geckos were observed twice daily for any changes in their general demeanor. The geckos in the control group were always visited and fed first to avoid cross-contamination.

Necropsy

Five geckos from each group, except for the last day, were euthanized at specified time intervals post-infection (PI) to evaluate their WNV status. Each gecko was euthanized using 200 mg/kg intramuscual Ketamine HCL (Ketaset®, Fort Dodge Animal Health, Fort Dodge, Iowa) followed by 0.05 ml intracardiac Beuthansia®-D Special solution (Schering-Plough Animal Health Corp., Union, New Jersey). Each gecko was placed into a pre-labeled self-sealing bag and transported to the Louisiana State University School of Veterinary Medicine (LSU-SVM) for processing. At necropsy, the brain, kidney, and spleen of each gecko were collected using sterile techniques and divided in two equal pieces. The tissue samples collected for viral isolation were placed into a 2.0ml screw top cryotube and stored at -70°C. The samples for RT-PCR were placed into 0.2 ml RNA*later*® (Ambion, Inc., Austin, Texas) and stored at -70°C. Geckos were tested by RT-PCR and culture since their small size (< 6.0 grams) precluded

ante-mortem testing. The geckos were necropsied at 2, 5, 10, and 15 days post inoculation. Table 8 shows the necropsy schedule for the geckos.

Table 8: Necropsy Schedule for Control and WNV Inoculated Mediterranean House Geckos

Date	Geckos in Group 1 (control)	Geckos in Group 2 (inoculated)
2 days PI, 6/29/03	1,2,3,4,5	24,25,26,36,37
5 days PI, 7/2/03	6,7,8,10,11	27,28,29,38,39
10 days PI, 7/7/03	12,13,14,15,16	30,31,32,40,41
15 days PI, 7/12/03	17,18,19,20,21,22,23	33,34,35,42,43

PI, days post-inoculation

Viral RNA Extraction

Reverse transcriptase polymerase chain reaction (RT-PCR) was used as a screening tool for WNV detection because it is a highly sensitive test. Extraction of viral RNA from the MHG was performed prior to testing by RT-PCR. Samples were extracted according to manufacturer's instructions using the RNeasy® Mini Kit (QIAGEN, Inc., Valencia, CA). Briefly, 20mg of each tissue (brain, kidney, and spleen) was transferred to an individual 1.5ml micro-centrifuge tube containing 600µl of buffer RLT. A stainless steel bead was added to each tube and homogenized for three minutes with a Retsch MM300 mixer mill (Retsch GmbH & Co, Hann, Germany). The tissue sample was then centrifuged (Eppendorf 5415R, Eppendorf North America, Inc., Westbury, NY) for three minutes at 13,000rpm. After the sample was centrifuged, 600µl of supernatant was then removed and added to 600µl of 70% ethanol. Once carefully mixed, 700µl of the sample was pipetted into an RNeasy mini column sitting in a 2ml collection tube. The sample was then centrifuged for 15 seconds at 13,000rpm with the flow-through being discarded after centrifugation. In a separate tube, 1120µl Buffer RDD and 160µl DNase were

mixed together by gently inverting the tube to produce DNase 1 incubation mix. The RNeasy column was transferred to a new collection tube where 350µl of Buffer RWI was added and centrifuged again at 13,000rpm for 15 seconds to wash the column. After discarding the flow-through, 80µl of DNase 1 incubation mix was then pipetted directly onto the spin column and incubated at room temperature for 15 minutes.

After the incubation period, 350µl of Buffer RWI was added to the column, which was then centrifuged for 15 seconds at 13,000rpm and the flow-through discarded after centrifugation. The column was then placed into a new collection tube where 500µl of Buffer RPE was added. Again the sample was centrifuged and the flow-through discarded. The column was then placed into a new collection tube where 500µl of Buffer RPE was added and the sample was centrifuged for two minutes at 13,000rpm. The RNeasy column was then transferred to a new 1.5 ml micro-centrifuge collection tube. Next, 30µl of RNase free water was added directly to the membrane and the sample was centrifuged at 13,000rpm for one minute. Finally, the spin column was discarded and the remaining sample was placed in the freezer at -70°C.

Reverse Transcriptase Polymerase Chain Reaction

The spleen, kidney, and brain tissues collected at necropsy were tested for viral RNA using RT-PCR. RT-PCR was performed using the Titan® One Tube RT-PCR system (Roche Molecular Biochemicals, Indianapolis, IN) in accordance with manufacturer's directions. In brief, a 50µl reaction was made using 3µl of sample RNA and 47µl of reaction master mix, which contained 1µM of forward and reverse primers.

The kidneys, brains and spleens were analyzed using primer set WN9483 (5'-CACCTACGCCCTAAACACTTTCACC-3') and WN9794 (5'-GGAACCTG-

CTGCCAATCATACCATC-3'), which are derived from nonstructural protein 5, and primer set WN212 (5'-TTGTGTTGGCTCTCTTGGCGTTCTT-3') and WN619 (5'-CAGCCGACAGCACTGGACATTC-ATA-3'), which are derived from the nucleocapsid and premembrane portion of the genome.[123] The PCR amplification was processed on a 9600 Thermal-cycler (Applied Biosystems, Foster City, CA). The thermal cycling consisted of 50°C for thirty minutes; 95°C for fifteen minutes; 40 cycles of 94°C for one minute, 65°C for two minutes, 72°C for one min and a final extension period of 72°C for ten minutes. A total of 12µl of RT-PCR product was analyzed on a 2% agarose gel in Tris-acetate EDTA and visualized with ethidium bromide and UV light transillumination. The RT-PCR procedure was repeated using a second set of primers,

Sequencing

Two samples, one control and one inoculated gecko, were genetically sequenced. Positive RT-PCR samples were cleaned using the MinElute PCR purification kit (QIAGEN, Inc., Valencia, California) according to manufacturer's instructions. Briefly, 75µl of Buffer PB were added to 15µl of PCR reaction and mix. Then the MinElute column was placed in a provided 2ml collection tube in a rack. Samples were added to the MinElute column and centrifuged at 13,000 rpm to bind DNA for one minute. The flow-through was then discarded and the MinElute column was placed back into the same tube. Buffer PE (750µl) was added to wash the product in each column. The columns were centrifuged for another minute at 13,000rpm. The flow-through was discarded and the column was spun for addition minute at 13,000rpm to remove remaining Buffer PE. The columns were placed into new 1.5ml microcentrifuge tubes and 10µl of Buffer EB was added to elute DNA. Columns were allowed to sit for one minute before they were

spun at 13,000rpm for a final time. Cleaned PCR products were sequenced using ABI Prism® 377 DNA sequencer (Applied Biosystems, Foster City, California) using WNV primer set 212-619.

Virus Isolation

Virus isolation was performed on individual gecko tissues that were positive for WNV by both sets of primers. Tissues from geckos that were RT-PCR negative by at least one set of primers or were RT-PCR negative were pooled and cultured together since they were considered negative for WNV. Tissues were added to 200µl of minimum essential media (MEM) plus 100µg/ml vancomycin, 100µg/ml gentamycin, 500µg/ml kanamycin, and 10µg/ml amphotericin B in 1.5 ml microcentrifuge tubes. Tissues were then homogenized for 30-60 seconds and 200µl more of the aforementioned media was added. Samples were then centrifuged at 4°C at 2000 rpm for ten minutes. Next, culture media was removed from culture vials containing Vero cells from the LSU-SVM Cell Culture Lab and 200µl of sample supernatant was added to each vial. Samples were run in duplicate, two vials per sample. The vials with inoculum were incubated at 37°C and 5% CO_2 for 90 minutes.

The inoculum was removed and vials were rinsed with 500µl of media containing MEM plus 200IU/ml penicillin, 100mg/ml streptomycin, 10µg/ml amphotericin B, and 5% fetal bovine serum. After the vials were rinsed, 1ml of the aforementioned media was added to each tube. Vials were placed in an incubator at 37°C and 5% CO_2. Vials were monitored daily for cytopathic effects (CPE). If no CPE was seen after 7 days, cultures were subcultured. Subculture was performed by taking 200µl of supernatant and cells from the cultures and placing it in new culture vials with Vero cells. Vials were

incubated, rinsed, and monitored for CPE as previously described. Brain tissue samples from two geckos (30 and 32) were not submitted for subculture. Samples showing CPE were confirmed by the VecTest™ (Medical Analysis Systems, Camarillo, CA) WNV antigen test and by RT-PCR.

Virus Isolation Confirmation

Samples that demonstrated CPE were extracted using the RNeasy method described above. Confirmation of virus isolation by RT-PCR was accomplished using the ABI Prism® 7900 (Applied Biosystems, Foster City, California) and the WNV E primer.[122] For the assay, 5µl of RNA from the sample was mixed with 1.45µl of RNase free water, 7.5µl of RT-PCR master mix (QuantiTect™ Probe RT-PCR , QAIGEN, Inc., Valencia, CA), 0.3µl of WNV E forward primer (5'-TCAGCGATCTCTCCACCAAAG-3'), 0.3µl of WNV E reverse primer (5'-GGGTCAGCACGTTTGTCATTG-3'), 0.3µl of (FAM-TGCCCGACCATGGGAGAAGCTC-BHQ1) probe, and 0.15µl of Quanitect RT Mix enzyme. The cycle was run as follows: thirty minutes at 50°C for reverse transcription, ten minutes at 95°C for initial activation, and forty cycles of fifteen seconds at 95°C and one minute at 60°C.

Statistical Analysis

Statistical analysis was performed using the StatCalc portion of Epi Info version 3.2.2 (Centers for Disease Control and Prevention, Atlanta, Georgia). StatCalc was used to construct 2x2 tables to compare the kidney, spleen, and brain RT-PCR results, as well as the culture results for the control and inoculated groups.

Results

To be considered RT-PCR positive, gecko tissues had to be positive by both sets of primers. Five (22%) of the brain samples from the control geckos were RT-PCR positive, while eight (40%) of the inoculated gecko brains were positive. None (0/23) of the spleen samples of the control geckos were RT-PCR positive, while ten (50%) of the inoculated gecko spleens were positive. One (4%) of the kidney samples from the control geckos was RT-PCR positive while five (25%) of the inoculated gecko kidneys were positive (Table 9).

Despite the high number of samples testing positive for WNV by RT-PCR, only six geckos became infected. Live virus was isolated from seven gecko samples in the inoculated group. The seven tissue samples were the spleens from five geckos (29, 31, 39, 40, and 43) the kidney from one gecko (43), and an undetermined tissue of one gecko (27) since kidney, spleen, and brain tissues were grouped together for culture since tissues were not positive by both sets of RT-PCR primers (Table 9). Viral isolates from these geckos were confirmed as WNV. None of the control geckos had positive cultures.

Of the five control geckos that were WNV positive, 4/5 (80%) only had one tissue positive, while 1/5 (20%) had two tissue (brain, kidney). Of the 14 inoculated geckos that were WNV positive by RT-PCR, 5/14 (35.7%) had a single tissue positive, 7/14 (50%) had two tissues positive, and 2/14 (14.3%) had all three tissues (brain, spleen, and kidney) positive.

The brain of gecko 10 (control group) and gecko 33 (inoculated group) were sequenced and came back as homologous for WNV.

Table 9: Reverse Transcriptase Polymerase Chain Reaction (kidney, spleen, and brain)and Culture Results For Control and WNV Inoculated Mediterranean House Geckos

Gecko	Group	Days PI	Kidney	Spleen	Brain	Culture
1	Control	2	Negative	Negative	Negative	Negative
2	Control	2	Negative	Negative	Negative	Negative
3	Control	2	**Positive**	Negative	**Positive**	Negative
4	Control	2	Negative	Negative	**Positive**	Negative
5	Control	2	Negative	Negative	**Positive**	Negative
6	Control	5	Negative	Negative	Negative	Negative
7	Control	5	Negative	Negative	**Positive**	Negative
8	Control	5	Negative	Negative	Negative	Negative
10	Control	5	Negative	Negative	**Positive***	Negative
11	Control	5	Negative	Negative	Negative	Negative
12	Control	10	Negative	Negative	Negative	Negative
13	Control	10	Negative	Negative	Negative	Negative
14	Control	10	Negative	Negative	Negative	Negative
15	Control	10	Negative	Negative	Negative	Negative
16	Control	10	Negative	Negative	Negative	Negative
17	Control	15	Negative	Negative	Negative	Negative
18	Control	15	Negative	Negative	Negative	Negative
19	Control	15	Negative	Negative	Negative	Negative
20	Control	15	Negative	Negative	Negative	Negative
21	Control	15	Negative	Negative	Negative	Negative
22	Control	15	Negative	Negative	Negative	Negative
23	Control	15	Negative	Negative	Negative	Negative
24	Inoculated	2	Negative	Negative	Negative	Negative
25	Inoculated	2	Negative	Negative	Negative	Negative
26	Inoculated	2	Negative	Negative	**Positive**	Negative
27	Inoculated	5	Negative	Negative	Negative	**Positive Tissue**
28	Inoculated	5	Negative	**Positive**	Negative	Negative
29	inoculated	5	Negative	**Positive**	**Positive**	**Positive Spleen**
30	Inoculated	10	**Positive**	**Positive**	**Positive**	Negative, no brain
31	Inoculated	10	Negative	**Positive**	**Positive**	**Positive Spleen**
32	Inoculated	10	**Positive**	**Positive**	**Positive**	Negative, no brain
33	Inoculated	15	**Positive**	Negative	**Positive***	Negative, brain contaminated
34	Inoculated	15	Negative	Negative	**Positive**	Negative
35	Inoculated	15	Negative	**Positive**	**Positive**	Negative
36	Inoculated	2	Negative	Negative	Negative	Negative
37	Inoculated	2	Negative	Negative	Negative	Negative
38	Inoculated	5	Negative	Negative	Negative	Negative
39	Inoculated	5	**Positive**	**Positive**	Negative	**Positive Spleen**
40	Inoculated	10	Negative	**Positive**	Negative	**Positive Spleen**
41	Inoculated	10	Negative	**Positive**	Negative	Negative
42	Inoculated	15	Negative	Negative	Negative	Negative
43	Inoculated	15	**Positive**	**Positive**	Negative	**Positive Spleen, Kidney**

PI, days post-inoculation; *Sequenced using primers 212-619.

Statistical analysis using StatCalc demonstrated significant differences in the results for spleen RT-PCR and culture results for the control and inoculated groups with a Fisher's Exact one-sided p-value of 0.0013 and 0.0029, respectively. The results for the kidney and brain RT-PCR between the two groups were not significantly different (p-value of 0.0724 and 0.5000, respectively).

Discussion

Early detection in areas of high viral activity plays an essential role in prevention and awareness programs for WNV. To date, mosquitoes and dead birds are the primary species being tested to monitor the presence of WNV in a particular region. It has not been shown whether reptiles can be used as consistent indicators of the relative exposure of a particular environment to the presence of WNV.

This study represents the first attempt to experimentally infect Mediterranean House Geckos with WNV. The finding of WNV or WNV RNA within the geckos was confirmed by multiple, independent techniques including RT-PCR, sequencing, and cell culture in both control and inoculated animals. The numerous positive WNV RT-PCR samples found, most of which could not be confirmed with culture, could possibly be attributed to circulation of viral RNA since RT-PCR can not distinguish between viable and non-viable virus. The use of two RT-PCR primer sets for WNV were used to designate a sample WNV positive thereby decreasing the chance misclassification by reducing the likelihood of false positives with RT-PCR. Sequencing of all WNV RT-PCR positive samples would have also decreased the chance of misclassification, but was not performed on more WNV RT-PCR positive samples due to financial constraints. However, at least one sample from both the control and inoculated groups were

sequenced, and both found to be homologous to WNV. This confirms that wild-caught Mediterranean house geckos are exposed to WNV.

The finding of a WNV culture positive gecko with negative RT-PCR results is interesting. This inoculated gecko did have its brain and spleen test RT-PCR positive by one set of primers. Serial testing decreases the sensitivity of the test since there is an increased risk that the causative agent could be missed. In regards to RT-PCR, a false negative could occur through the addition or subtraction of nucleic acid or the mishandling of the sample.

The finding of five WNV RT-PCR positive controls is also very interesting. It is believed that the controls arrived to the facility naturally infected with WNV, since cross-contamination between control and inoculated geckos was minimized by the use of separate rooms with separate ventilation and by visiting inoculated geckos first. However, this could not be confirmed by ante-mortem testing because of the small size (< 6.0 grams) of the geckos. The negative culture results most likely stems from RT-PCR detecting WNV RNA since it is unknown when they became exposed. In a study with orally infected alligators held at 27°C, alligators were viremic for 14 days post-infection.[40] This report agrees with one of the geckos being culture positive 15 days post-inoculation. Sequencing of RT-PCR products from one of the control geckos found the sequence to be nearly identical to the sequence obtained from one of the inoculated geckos.

It is not surprising that the inoculated group would yield more culture positives than the control group since they were administered live virus. It is surprising that there is a difference between the groups in regards to spleen RT-PCR results. This is likely

due to the spleen, a large lymphoid organ, trapping and concentrating foreign substances, such as WNV, carried in the bloodstream.

Findings reported here suggest that these insectivorous lizards may indeed play a role in the epidemiology of WNV since geckos became infected with an oral inoculum of WNV. Because these reptiles are insectivores, and mosquitoes can harbor quantities of WNV similar to the inoculum, it is feasible that oral ingestion by this insectivore may serve as a source of infection for humans.[51] Future study is needed to prove this hypothesis.

This study demonstrated that geckos produced a viremia as evidenced by the isolation of WNV in culture although the viremia was not quantified. Information on the quantity of virus would be useful in determining if geckos are capable of producing a sufficient viremia capable of infecting a mosquito. In many instances, animals can produce a viremia, but it is usually insufficient to infect a mosquito thus decreasing the likelihood that a species serves a significant amplifying host.[51,99,100]

To be considered an amplifying host, hosts must not only become infected with sufficient viremias to infect a vector, they must also demonstrate they can become infected in the wild and also come into ample contact with vectors to transmit WNV. The study reported here demonstrated that Mediterranean house geckos can become infected with an oral dose of WNV, but further study on WNV in naturally infected geckos is warranted to determine the prevalence of WNV in wild-caught geckos to evaluate the role of geckos in the epidemiology of WNV as a potential amplifying host.

THE PREVALENCE OF WEST NILE VIRUS IN WILD CAUGHT MEDITERRANEAN HOUSE GECKOS (*HEMIDACTYLUS TURCICUS*) FROM ENDEMIC AREAS IN EAST BATON ROUGE PARISH, LOUISIANA

Introduction

Previous research has shown ectotherms may be suitable hosts for arbovirus infections. Field studies have found antibodies to Japanese encephalitis (JE) virus in a variety of snakes, lizards, and turtles in Asia.[40,116,117] Laboratory studies have found that JE virus can replicate in lizards and snakes in Asia.[116,117] Other laboratory experiments with garter snakes (*Thamnophis* spp.) and Texas tortoises (*Gopehrus berlandieri*) in the United States showed they were competent reservoirs for Western equine encephalitis virus.[116,118] Finally, St. Louis encephalitis (SLE) virus neutralizing antibodies were detected in a painted turtle (*Chrysemys picta picta*) and a leopard frog (*Rana pipiens pipiens*) in New York.[116]

Historically, reptiles and amphibians have not been considered to play an important role in the transmission of WNV in endemic areas in the eastern hemisphere or in its new geographic distribution zone in the western hemisphere. However, few studies have truly evaluated the role of reptiles or amphibians in the dissemination of the WNV. One of the earliest studies revealed that the lake frog (*Rana ridibunda*) in Russia can develop a viremia capable of infecting mosquitoes.[19,24] WNV antibodies have also been detected in Nile crocodiles (*Crocodylus niloticus*).[24,40] An experimental inoculation trial in the United States with bullfrogs (*Rana catesbeiana*), Florida garter snakes (*Thamnophis sirtalis sirtalis*), and green iguanas (*Iguana iguana*) showed that they became infected, but only the bullfrogs and the iguanas developed a viremia.[116] Recently, WNV has been isolated from farmed alligators in Florida, Georgia, and

Louisiana.[40,41] The affected alligators were found to have clinical signs consistent with encephalitis. The alligators were also found to produce significant viremias, suggesting that these animals could serve as amplifying hosts. One human case of WN fever has been linked to an outbreak of WNV in juvenile American alligators (*Alligator mississipiensis*).[40,41]

The Mediterranean house gecko (MHG) (*Hemidactylus turcicus*) is a nocturnal squamate that is intimately associated with human dwellings. This species of gecko originated in Europe, but was introduced into North America through Florida in the early 1900's.[120] Since that time, the lizard has spread throughout the Southeastern United States, including Louisiana. These animals are routinely found in large numbers on human domiciles in southeastern Louisiana. If these animals play a role in the epidemiology of WNV, it would be important to characterize the level they are involved. Geckos are insectivores and are frequently found preying on insects that congregate around artificial light sources. Because these reptiles ingest various arthropods, they could serve as a vector/reservoir for arthropod-borne diseases. Experimental studies in birds and American alligators (*Alligator mississippiensis*) have demonstrated that WNV can occur through oral ingestion.[40,51] The purpose of this study was to estimate the prevalence of WNV in MHG from WNV endemic areas in East Baton Rouge Parish, Louisiana.

Methods and Materials

A cross-sectional study was conducted to estimate the prevalence of WNV in MHG from endemic areas in East Baton Rouge (EBR) Parish, Louisiana. Geckos were captured at three locations, Lazy B Stables (30.41322 °N, -91.00003 °W), Highland Road

Park (30.35185 °N , -91.07150 °W), and City Park (30.43280 °N, -91.17032 °W), within the parish. These locations were selected because they had been used as arbovirus surveillance sites by the East Baton Rouge Mosquito Abatement and Rodent Control (EBRMARC), and were known to have WNV. Geckos were captured by hand or using a fish net after 20:00 hours between April and October of 2004. Geckos were not pursued after October because they were difficult to find when temperatures decreased. Captured geckos were promptly placed into individual plastic containers. Time and location were recorded for each animal. After capture, geckos were transported to LSU-SVM for euthanasia. Upon arrival, geckos were given 0.05mls of Ketamine HCL (Ketaset®, Fort Dodge Animal Health, Fort Dodge, Iowa) intramuscularly followed by 0.05mls of intracardiac Beuthanasia®-D Special solution (Schering-Plough Animal Health Corp., Union, New Jersey). Upon euthanasia, geckos were placed into an individual plastic bag, weighed, and stored at 40°C.

Necropsy

MHG were necropsied the day after capture in a biosafety level 2 (BSL-2) cabinet. Geckos were measured and sexed as they were put into the cabinet on a pad. Geckos were necropsied using aseptic techniques. New or sterilized instruments and clean absorption pads were used for each gecko. The abdomen and thorax were incised using sterile scissors. The spleen was then removed with disposable forceps. After removal, the spleen was cut in half with one piece submitted for the reverse transcriptase polymerase chain reaction (RT-PCR) and the other piece for culture. Tissues were placed placed into a 1.5ml conical screw top cryovials. Approximately 200µl of RNA*later*® (Ambion, Inc., Austin, Texas) was added to the cryovial tube with tissue for RT-PCR.

After the spleen was removed, geckos were further dissected to expose the kidneys. Forceps and scissors were used to remove one kidney and place it in the same vial as spleen tissue for testing by RT-PCR, and the other kidney removed and placed in a separate vial for culture. Similarly, half of the brain of placed into the vial for RT-PCR testing, while the other half was placed into a separate tube for culture. Tissues for RT-PCR and for culture were stored at -70°C prior to testing. Geckos were tested for WNV by RT-PCR and culture since their small size (< 6.0 grams) precluded them from ante-mortem testing.

Viral RNA Extraction

RNA extraction was performed in the following manner. Briefly, 250µl of BA-1 diluent, a metallic BB, and gecko brain, spleen, and kidney tissue were placed into a 1.5ml microcentrifuge tube. Next 750µl of Trizol® (Invitrogen, Carlsbad, California) was added to the tubes. Tubes were then placed in a Retsch MM300 mixer mill (Retsch GmbH & Co, Hann, Germany) for three minutes to homogenize samples. After tissue homogenization, one milliliter of homogenate was transferred to a new microcentrifuge tube. The samples were then incubated at room temperature for five minutes. Next, 200µl of chloroform were added to the samples. Tubes were then capped and shook vigorously by hand for fifteen seconds. Samples were incubated at room temperature for another ten minutes. After incubation, samples were centrifuged (Eppendorf 5415R, Eppendorf North America, Inc., Westbury, NY) at 12,000x g for fifteen minutes at 2-8ºC. Next, the aqueous phase was transferred into new microcentrifuge tubes. After the transfer of the aqueous phase, 500µl of isopropyl alcohol was added to precipitate the

RNA. Samples were then mixed well. After mixing, sample were incubated the samples at room temperature ten minutes.

Tubes were then capped and centrifuged at 12,000 g for ten minutes at 2-8ºC. Next, the aqueous phase was removed while the RNA pellets remained in the tubes. RNA pellets were washed with 1ml of 75% ethanol and mixed by vortexing. Vortexing dislodged the RNA pellets, but did not dissolve the RNA pellets. After vortexing, the samples were centrifuged at 7500g for five minutes at 2-8ºC. The samples were then stored in a -20ºC freezer until all the samples were processed. After all the samples were processed, the ethanol was poured off and the RNA pellets were allowed to air dry for 5-10 minutes. After drying, 50µl of DEPC-water was added to each tube and the RNA pellet for each sample was dissolved by passing it through the solution a few times using the pipette tip. Finally, samples were placed in a heat block for 10 minutes at 55-60ºC before they were tested for viral RNA by RT-PCR.

Reverse Transcriptase Polymerase Chain Reaction

Samples were tested using the ABI Prism® 7900 (Applied Biosystems, Foster City, CA) and the WNV Envelope (E) primer.[124] The assay was performed using the Quantitect™ Probe RT-PCR kit (QIAGEN, Inc., Valencia, CA) according to manufacturer's instructions. For the assay, 5µl of RNA from the sample was mixed with 1.45µl of RNase free water, 7.5µl of Quantitect RT-PCR master mix, 0.3µl of WNV E forward primer (5'-TCAGCGATCTCT-CCACCAAAG-3'), 0.3µl of WNV E reverse primer (5'-GGGTCAGCACGTT-TGTCATTG-3'), 0.3µl of FAM-TGCCCGACCATGGGAGAAGCTC-BHQ1 probe, and 0.15µl of Quantitect RT Mix enzyme. The cycle was run as follows: thirty minutes at 50°C for reverse transcription,

ten minutes at 95°C for initial activation, and then forty cycles of fifteen seconds at 95°C and one minute at 60°C were ran.

Virus Isolation

Tissues that were positive by RT-PCR were removed from storage and immediately added to 200µl of MEM plus 100µg/ml vancomycin, 100µg/ml gentamycin, 500µg/ml kanamycin, and 10µg/ml amphotericin B in 1.5ml microcentrifuge tube for virus isolation. Tissues were then homogenized for 30-60 seconds. After the tissues were homogenized, 200µl more of the aforementioned media was added. Samples were then centrifuged at 4°C at 2000 rpm for ten minutes. Next, media was drained from culture vials containing Vero cells from the LSU-SVM cell culture lab and 200µl of sample supernatant was added to individual vials. Samples were run in duplicate, two vials per sample in case of contamination. The inoculated vials were incubated at 37°C and 5% CO_2 for 90 minutes. The inoculum was removed and vials were rinsed with 500µl of media containing MEM plus 200IU/ml penicillin, 100mg/ml streptomycin, 10µg/ml amphotericin B, and 5% fetal bovine serum. After the vials were rinsed, 1ml of the rinse media was added to each tube. Vials were placed in an incubator at 37°C and 5% CO_2. Vials were monitored daily for cytopathic effects (CPE). If no CPE was seen after seven days, cultures were subcultured. Subculture was performed by taking 200µl of supernatant and cells from the cultures and placing them in new individual culture vials with Vero cells. Vials were incubated, rinsed, and monitored for CPE as previously described for an additional seven days.

Results

Of the 134 geckos captured, 66 (49.3%) were caught at three different buildings at City Park, 54 (40.3%) were caught at two buildings at Highland Road Park, and 14 (10.4%) were caught at three buildings on the Lazy B Stables premise. May and July yielded the most captures with 28 (20.2%) each month. Females were caught more often (66/134, 49.3%) than males (56/134, 41.8%), and 12 geckos (8.9%) were recorded as gender unknown. WNV was detected by RT-PCR in only 3/134 geckos (2.2%; CI: 0, 4.7). All three geckos (34, 53, and 109) that were positive by RT-PCR, had negative culture and subculture results. Gecko 34 was caught in May at Lazy B Stables, gecko 53 was caught in June at Highland Road Park, and gecko 109 was caught in September at City Park.

Discussion

Despite the high number of captures, only three geckos (2.2%) demonstrated the presence of WNV RNA, which is lower that the number of wild-caught geckos (22.7%) that tested RT-PCR positive in an inoculation study performed at LSU-SVM. In this study, every attempt was made to minimize WNV transference between geckos at all stages of the study including capture. Disposable gloves were changed after each gecko was caught and geckos were housed separately in plastic containers. The same cannot be said about the geckos used in the inoculation study since they were acquired from a reptile collector.

Genetic sequencing of the three positive geckos in this study was attempted to further confirm the presence of WNV, but RT-PCR products analyzed on a 2% agarose gel in Tris-acetate EDTA and visualized with ethidium bromide and UV light

transillumination were negative. This could be due to the sensitivity of real time RT-PCR compared to traditional or gel-based RT-PCR methods, or it could be related to the deterioration of extracted RT-PCR products after being held at -70°C for an extended period of time (greater than 1 year). Extractions were also performed on the culture and subculture supernatants to try to obtain a product for sequencing, but these samples were also negative for WNV using traditional RT-PCR methods. This was likely due to negative culture and subculture results.

The lack of an additional test confirming the presence of WNV in these wild-caught geckos may be attributed to several factors. First, the small size of these geckos ($\leq$ 6.0 grams) precludes them from most serological testing for WNV antibodies or antigen limiting what tests can be performed on them. It is also possible the viremia present in the geckos was gone, and that only WNV RNA could be detected by RT-PCR.

MGH are inherently associated with human domiciles. The urban location of these animals, in addition to their feeding habits (insectivores), suggests they are likely exposed to WNV. The finding of a low prevalence of WNV in these animals should not be construed to suggest that exposure is low, as this study measured viral RNA. Therefore, it is likely exposure is higher than reported here. To measure exposure, a serologic test measuring antibody levels would be necessary. Unfortunately, the small size of these animals precluded our ability to collect sufficient sample for this test.

The results of this study do suggest that MHG are unlikely to serve as important role an amplifier of WNV, but could potentially serve as a natural biologic control method for WNV infected mosquitoes since they did not develop clinical signs when orally inoculated and have the ability to eat over 100 mosquitoes per day.[125] Anecdotally,

it is not uncommon to hear complaints by homeowners about the presence of these animals. Historically, mosquito control in the United States, and more specifically Louisiana, is done by spraying chemicals. Although these chemicals are considered not harmful to humans, their overall impact on the environment has not been evaluated. By promoting the use of natural mosquito control methods, such as natural predators, public health officials can reduce the risk of human exposure to WNV.

CONCLUSIONS

In a short period of time, WNV has gone from an established Old World disease to becoming the premiere example of how a disease can find a new niche in a new geographic setting. WNV has firmly established itself as one of the most common arboviral diseases in North America. The more we learn about the epidemiology of WNV, the greater the potential for disease prevention in both humans and animals. The introduction of WNV into the New World has afforded scientists, health officials, and others the opportunity to study a new emerging disease. It has strengthened the bond between the veterinary and human medical fields making these groups realize what impacts one also impacts the other. It has also forced the development of new diagnostic and surveillance techniques that will hopefully prepare and aid us in future disease introduction.

Along with its quick geographic spread, WNV has produced several surprising findings. One, new modes of transmission have been associated with the spread of WNV in North America. Infections have occurred through blood transfusions, organ transplants, breast milk, and placenta.[4] As a result of these findings, blood donations are now screened for WNV. Another interesting phenomenon is the scope of species affected by WNV. Evidence of WNV infections have been documented in numerous species including squirrels, reindeer, bats, bears, and other animals.[50] WNV has even been responsible for die-offs at alligator farms.[40,41] Still other research has shown that multiple mosquito hosts are competent WNV vectors. These findings indicate that WNV is a complex biological agent that still has many lessons to teach in order to prevent human and animal morbidity and mortality.

Like other arboviruses, WNV is dependent mostly on an insect vectors for replication and development as well as transmission between to vertebrate hosts. In this instance, the mosquito vectors of greatest importance in the United States are members of the genus *Culex*. Mosquitoes in turn are influenced by abiotic factors such as temperature and precipitation. Temperature affects the life cycle of the mosquito while precipitation affects mosquito abundance. These factors also affect the extrinsic incubation period, the time for the development and transmission of the virus itself.[69,70] Due to the very nature of vector and the virus, environmental influences are hypothesized to affect WNV transmission.

As part of this study, environmental predictive models using geographic information systems and remote sensing in relationship to bird and human data from 2002 and 2003 were constructed. Results indicate that environmental variables, especially those related to hydrology and temperature, do influence the transmission and development of WNV, but it may not fit a consistent pattern since 2002 and 2003 bird and human models did not fit the same statistically. Statistical analysis showed the best model to be a 2003 human model with 13 variables that explained 74% of the variation in human prevalence by parish. Statistical analysis also indicated that WNV human cases and WNV dead birds are a product of an urban WNV cycle. These results can be used to guide mosquito abatement and public health officials in detecting WNV hotspots and implementing appropriate control measures. Further refinement and testing against dead bird and human environmental factors from 2004 and 2005 is needed to validate the model developed in the present study.

One limitation of the analysis was obtaining geographic information for records. In 2002, only 80.5% of the dead birds could be geocoded and this included matching some records to zip codes only. The lack of geographic information precluded the use of horse data, mosquito data, and sentinel chicken in development of a predictive model. This data would have been useful in identifying other WNV cycles and transmission factors, and in refining the models. Hopefully, future changes will be made in these records to incorporate geographic information since this can also be used to locate hot spots and examine other data sources that may define the spatial and temporal transmission of WNV.

In "The Arboviruses: Epidemiology and Ecology", Michael B. Gregg writes, "An important adjunct to or surrogate for human arbovirus disease surveillance is the monitoring of nonhuman vertebrate hosts and vector species. Humans usually represent an incidental or dead-end host, generally have a high rate of inapparent to apparent infections, and contribute insignificantly to the zoonotic cycle."[11] This is the reason wild bird surveys are important in understanding the epidemiology of WNV.

Through the assistance of East Baton Rouge Mosquito Abatement and Rodent Control (EBRMARC), it was possible to conduct a serosurvey in wild birds from November 2002 through October 2004 at locations that were known to have arboviral activity. In this study, 1287 samples from almost 49 bird species at 41 locations were tested for WNV antibodies by the plaque reduction neutralization. Of these, 222 (17.25%, CI: 15.19, 19.31) samples were positive for WNV antibodies while five birds were positive for Saint Louis encephalitis virus and a Hermit Thrush had an ambiguous flavivirus result . Analysis showed differences between locations, species, age, sex, and

month with northern cardinals (*Cardinalis cardinalis*) implicated as one of the main WNV hosts and a higher prevalence reported in adult birds. The finding of northern cardinals, a peridomestic bird species, as one of the main WNV hosts agrees with the GIS finding that the WNV cycle in Louisiana occurs in an urban setting. The higher adult seroprevalence can be explained in part by antibody duration which can cause adults to be positive for multiple years.[114] In order to understand the current WNV situation, one must consider testing juvenile birds and/or testing for viral antigen since testing for viral antibodies in adults can inflate the seroprevalence and can not provide information on when or where a bird was infected.

Mediterranean house geckos (*Hemidactylus turcicus*) were also evaluated as potential reservoirs of WNV since they are insectivores and commonly found around human domiciles. Experimental inoculation showed they could become infected with an oral dose of WNV, but wild caught geckos demonstrated a low WNV prevalence (3/134, 2.2%; CI: 0, 4.7) as well as an almost undetectable viremia. These results are consistent with other experimental trials that demonstrate that animals can become infected through an artificial means with WNV, but it does not carry over well to what is observed in naturally infected animals.[88,98-100]

Overall, these findings indicate that WNV is endemic in Louisiana and in East Baton Rouge Parish. These studies here have presented important information on the transmission and epidemiology, but gaps in the basic knowledge base still exist. Further study is needed to fill in these gaps and can be accomplished by a local GIS project. This could be done for East Baton Rouge Parish using the locations for the WNV wild bird survey, location information, and mosquito pool results for the same locations. This GIS

project could provide information on the microhabitat and how it affects the WNV host and vector cycle.

Additionally, mosquito abatement districts and public health officials need to re-evaluate their control and prevention programs since serology results and GIS results indicate the main WNV cycle is occurring in urban settings such as the cities of Baton Rouge and Shreveport. Residents need to be informed that they are at risk of being infected with WNV in their backyards and need to take precautions such as emptying water containers and wearing mosquito repellant to decrease their risk of WNV infection. For an even better understanding of the urban influence and local factors relating to transmission of WNV in backyards, it would be ideal to sample humans, birds, and mosquitoes at the same location.

Also, surveillance efforts using live birds should concentrate on using sentinel chickens or juvenile birds since antibody persistence can confound results and cause misclassification of current WNV activity. Another suggestion would be to test for WNV antigen instead of WNV antibodies since birds are viremic for a short period of time.

The studies presented here that indicate that Mediterranean house geckos can be infected with WNV, but are not reservoirs for WNV since they have an extremely low prevalence of natural WNV infection. One would suspect that they might be used as a natural means, as opposed to pesticides, in controlling WNV infected mosquito populations since no clinical signs were observed after experimental infection despite ingesting an oral dose of WNV equivalent to an infectious mosquito.

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APPENDIX A: MEDIA

BA-1 Diluent:

- 1X 199
- 1Molar Tris-HCL
- 1% bovine serum albumin
- 7.5% sodium bicarbonate
- 100X antibiotic antimycotic solution

Methyl Cellulose Overlay (2% methyl cellulose, 2% fetal calf serum):

Solution A (methyl cellulose mixture)

- 500 ml of glass-distilled water
- 20 grams methylcellulose powder

Solution B (2x MEM)

- 9.6 grams of powdered MEM or 9.98 grams of powdered DMEM
- 300 ml of glass-distilled water
- 5.95 grams of powdered HEPES buffer or 3.7 grams of powdered bicarbonate
- 1 N NaOH
- QS with glass-distilled water to make 500 milliliters
- 10 ml of Penicillin-Streptomycin solution
- 20 ml of sterile fetal calf solution

2X M199-E Overlay Media

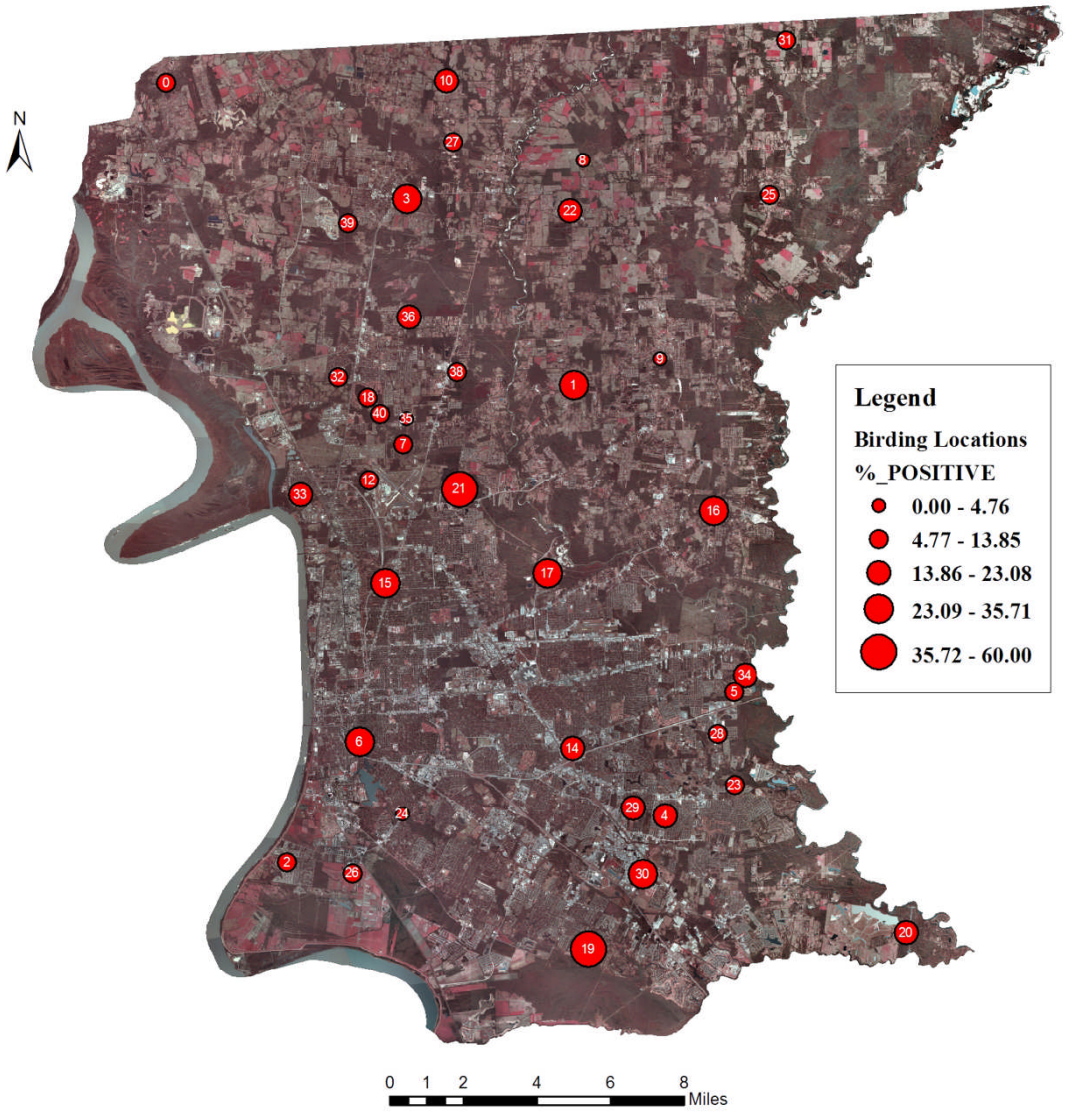
- 200 ml of 10X M199-Earle's BSS
- 40 ml heat inactivated fetal bovine serum
- 20 ml Penicillin-Streptomycin solution
- 20 ml L-Glutamine
- 2 ml Fungizone
- 2 ml Gentamycin
- QS with sterile water to 1 liter

APPENDIX B: TABLE OF BIRD SPECIES AND THEIR SCIENTIFIC NAMES

Species	**Scientific Name**
American Goldfinch	*Carduelis tristis*
American Redstart	*Setophaga ruticilla*
American Robin	*Turdus migratorius*
Blue Grosbeak	*Guiraca caerulea*
Blue Jay	*Cyanocitta cristata*
Brown Thrasher	*Toxostoma rufum*
Brown-headed Cowbird	*Molothrus ater*
Carolina Chickadee	*Poecile carolinensis*
Carolina Wren	*Thryothorus ludoviciantus*
Chipping Sparrow	*Spizella passerina*
Common Grackle	*Quiscalus quiscula*
Common Yellowthroat	*Geothylpis trichas*
Eastern Phoebe	*Sayornis phoebe*
European Starling	*Sturnus vulgaris*
Field Sparrow	*Spizella pusilla*
Gray Catbird	*Dumetella carolinensis*
Hairy Woodpecker	*Picoides villosus*
Hermit Thrush	*Catharus guttatus*
Hooded Warbler	*Wilsonia citrina*
House Finch	*Carpodacus mexicanus*
House Sparrow	*Passer domesticus*
Indigo Bunting	*Passerina cyanea*
Louisiana Waterthrush	*Seiurus motacilla*
Mourning Dove	*Zenaida macroura*
Northern Cardinal	*Cardinalis cardinalis*
Northern Junco	*Junco hyemalis*
Northern Mockingbird	*Mimus polyglottos*
Orchard Oriole	*Icterus spurius*
Pine Warbler	*Dendroica pinus*
Prothonotary Warbler	*Protonotaria citrea*
Purple Martin	*Progne subis*
Red-bellied Woodpecker	*Melanerpes carolinus*
Red-headed Woodpecker	*Melanerpes erythrocephalus*
Red-wing Blackbird	*Agelaius phoeniceus*
Rufous-throated Solitare	*Myadestes genibarbis*
Ruby Crowned Kinglet	*Regulus calendula*
Rufous-sided Towhee	*Pipilo erythrophthalmus*
Savannah Sparrow	*Passerculus sandwichensis*
Swainson's Warbler	*Limnothlypis swainsonii*
Tufted Titmouse	*Baeolophus bicolor*
White-throated Sparrow	*Zonotrichia albicollis*
White-eyed Vireo	*Vireo griseus*
Wilson's Warbler	*Wilsonia pusilla*
Wood Thrush	*Hylocichla mustelina*
Yellow-rumped Warbler	*Dendroica coronata*
Yellow-bellied Sapsucker	*Sphyrapicus varius*
Yellow-breasted Chat	*Icteria virens*

Unknown and Unknown Yellow-rumped Warbler removed from list

APPENDIX C: MAP OF BIRD TRAP SITES



Key: 0=Bickham Rd, 1=Blackwater Rd, 2=BREC Horse Center, 3=Carpenter Rd, 4=Cedar Ridge Park, 5=Centurion Place, 6=City Park, 7=Clark St, 8=Comite Dr, 9=Denham Rd, 10=Doyle Bayou Park, 11=Drusilla Park, 12=East Baton Rouge Mosquito Abatement and Rodent Control, 13=Ednie Dr, 14=Emmett Bourgeois Rd, 15=Evangeline Fire Station, 16=Greenwell Springs Park, 17=Greenwell Springs Rd, 18=Greenwood Park, 19=Highland Rd Park, 20=Hoo Shoo Too Rd, 21=Hooper Rd Park, 22=Hwy 64 at Blackwater Rd, 23=Lazy B Stables, 24=Lee High School, 25=Liberty Rd, 26=LSU Swine Unit, 27=MacHost Rd, 28=O'Neal Lane, 29=Parklawn Park, 30=Pecue Lane, 31=Riley Rd, 32=S. Magnolia Park, 33=Southern University, 34=Strain Rd, 35=Sunshine Rd, 36=Tristian Ave Park, 37=Welcome Heights, 38=While Bayou, 39=Willow Dr, 40=Zoo Entrance

VITA

Karen Ruth Gruszynski was born to Howard and Phyllis Gruszynski in Milwaukee, Wisconsin, on August 12, 1975. She graduated from Pius XI High School in 1993. She attended the University of Minnesota-Twin Cities and graduated with a Bachelor of Science in science in agriculture emphasis animal science in 1998. In 2001, she graduated from the University of Wisconsin-Madison with a Doctor of Veterinary Medicine. She briefly worked as a research associate at the University of Wisconsin-Madison and as a contractor for the National Wildlife Health Center in Madison, Wisconsin before starting the next phase of her education. In January 2002, she started pursuing a Doctor of Philosophy in Veterinary Medical Sciences at Louisiana State University in Baton Rouge, Louisiana. In the spring of 2004, she started a Master in Public Health at Louisiana State University Health Sciences Center in New Orleans, Louisiana. In May 2006, she graduated with her Master in Public Health from LSU Health Sciences Center. Her Doctor of Philosophy degree was confirmed in December 2006.